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Activation of human B lymphocytes. VIII. Differential radiosensitivity of subpopulations of lymphoid cells involved in the polyclonally-induced PFC responses of peripheral blood B lymphocytes.

Fauci AS, Pratt KR, Whalen G.

The differential effect of various doses of irradiation on subpopulations of human peripheral blood lymphoid cells involved in the pokeweed mitogen (PWM) induced PFC response against sheep red blood cells (SRBC) was studied. The plaque forming B cells were quite sensitive to low doses of irradiation with complete suppression of responses at 300 to 500 rad. On the contrary, helper T-cell function was resistant to 2000 rad. Co-culture of irradiated T cells with autologous or allogeneic B cells resulted in marked enhancement of PFC responses consistent with the suppression of naturally occurring suppressor cells with a resulting pure helper effect. Irradiated T-cell-depleted suspensions failed to produce this effect as did heat killed T cells, whereas mitomycin C treated T cells gave effects similar to irradiated T cells. These findings are consistent with a lack of requirement of cell division for a T-cell helper effect and a requirement of mitosis or another irradiation sensitive, mitomycin C sensitive process for a T-suppressor cell effect. These studies have potential relevance in the evaluation of subpopulations of human lymphoid cells involved in antibody production in normal individuals and in disease states.

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Activation of human B lymphocytes

VIII. DIFFERENTIAL RADIOSENSITIVITY OF SUBPOPULATIONS OF LYMPHOID CELLS INVOLVED IN THE POLYCLONALLY-INDUCED PFC RESPONSES OF PERIPHERAL BLOOD B LYMPHOCYTES

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Received 17 *November* 1977; *accepted for publication* 20 *January* 1978

Summary. The differential effect of various doses of irradiation on subpopulations of human peripheral blood lymphoid cells involved in the pokeweed mitogen (PWM) induced PFC response against sheep red blood cells (SRBC) was studied. The plaque forming B cells were quite sensitive to low doses of irradiation with complete suppression of responses at 300 to 500 rad. On the contrary, helper T-cell function was resistant to 2000 rad. Co-culture of irradiated T cells with autologous or allogeneic B cells resulted in marked enhancement of PFC responses consistent with the suppression of naturally occurring suppressor cells with a resulting pure helper effect. Irradiated T-cell-depleted suspensions failed to produce this effect as did heat killed T cells, whereas mitomycin C treated T cells gave effects similar to irradiated T cells. These findings are consistent with a lack of requirement of cell division for a T-cell helper effect and a requirement of mitosis or another irradiation sensitive, mitomycin C sensitive process for a T-suppressor cell effect. These studies have potential relevance in the evaluation of subpopulations of human lymphoid cells involved in antibody production in normal individuals and in disease states.

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INTRODUCTION

It is now well established that T lymphocytes exert critical regulatory influences on B-cell function in both animal and human systems (Gershon, 1974; Katz & Benacerraf, 1972; Waldmann & Broder, 1977). Distinct subpopulations of T cells manifesting helper or suppressor function have been identified in the mouse (Cantor & Boyse, 1975), and most recently Moretta, Webb, Grossi, Lydyard & Cooper (1977) have demonstrated functionally distinct subpopulations of human T cells which can be identified by the presence of an Fc receptor for either IgM (helper T cell) or IgG (suppressor T cell).

It is generally agreed that subpopulations of mononuclear cells differ in their sensitivity to ionizing radiation (Anderson & Warner, 1976). In that regard, using a system of pokeweed mitogen (PWM)-driven immunoglobulin production by human B cells, Moretta *et al.* (1977) have shown that their suppressor T cells were sensitive to radiation while helper T cells were radioresistant. In addition,

it has been demonstrated that co-culturing of irradiated purified human peripheral blood T cells with autologous or allogeneic B cells resulted in a significant enhancement of immunoglobulin production suggesting a selective inhibitory effect of radiation on suppressor as opposed to helper T-cell function (Siegal & Siegal, 1977).

Using a recently described haemolysis-in-gel plaque forming cell (PFC) assay to measure PWM-induced antibody production against sheep red blood cells (SRBC) by human peripheral blood B cells (Fauci & Pratt, 1976a), we have shown this system to be dependent on T cells (Fauci, Pratt & Whalen, 1976) and to operate by a balance between helper and suppressor phenomena (Haynes & Fauci, 1977; Fauci *et al.*, 1977).

In the present paper, we demonstrate the comparatively exquisite radiosensitivity of the plaque-forming B cell compared to the relative radioresistance of the helper function of unfractionated mononuclear or purified T-cell suspensions in this assay.

MATERIALS AND METHODS

Cell suspensions

Heparinized venous blood was obtained from normal adult donors and mononuclear cell suspensions were obtained by standard Hypaque-Ficoll density centrifugation. Mononuclear cell suspensions that were either enriched or depleted of T lymphocytes were obtained by sheep erythrocyte (E) rosetting of lymphocytes followed by separation of rosetted and non-rosetted cells by centrifugation over Hypaque-Ficoll gradients as previously described in detail (Fauci *et al.*, 1976). T-cell enriched suspensions generally contained between 95% and 100% T cells, while T-cell depleted suspensions contained less than 0·5% T-cells with approximately 45 to 53% B cells and an enrichment of monocytes to approximately 40 to 45%. Monocytes and subpopulations of lymphocytes were identified by previously described techniques (Fauci *et al.*, 1976).

Irradiation of cell suspensions

In various experiments, either unfractionated mononuclear cell suspensions, T-cell enriched or T-cell depleted suspensions were irradiated with a wide dose range of *X*-irradiation from 25 rad up to 10,000 rad. The source of radiation was a Philips 250 kVp dual head *X*-ray system.

Following radiation, cells were washed once in RPMI-1640 media (Grand Island Biological Co., Grand Island, NY) and resuspended in fresh media for culture as described below.

Mitomycin C treatment and heat killing

In certain experiments, cell suspensions were either pretreated with mitomycin C (Sigma Chemical Co., St Louis, MO), 40 μg/ml for 45 min at 37° or were heated at 56° for 45 min.

Cell cultures

Culture conditions for the generation of anti-SRBC PFC responses following polyclonal activation of human peripheral blood lymphocytes with PWM have been described in detail (Fauci & Pratt, 1976a). Briefly, cells were cultured in RPMI-1640 containing 1% trypticase soy broth, 2 mM L-glutamine, 100 u of penicillin per ml, 100 μg of streptomycin sulphate per ml, and supplemented with 10% pooled human AB or A serum absorbed twice with SRBC. Cultures were carried out in 12 × 75 mm plastic tubes (Falcon Plastics, Oxnard, CA) at a density of 2×10^6 cells in 1 ml. Cultures were incubated on a rocker platform (7 cycles/min) for 6 to 7 days at 37° in 5% CO_2 in air at 100% humidity. Cultures were stimulated either with PWM in a wide concentration range (1/20 through 1/10,000 final dilution) or media alone as control (background PFC). In experiments in which irradiated cells were co-cultured with unirradiated cells, 1×10^6 cells of each suspension were cultured to keep the cell density constant at 2×10^6 cells in 1 ml. The same was done in co-cultures with mitomycin C treated or heat killed cells.

Data are expressed as PFC per 10^6 cells. In certain of the co-culture experiments where indicated, data are expressed as expected PFC response per 10^6 cells compared to observed PFC response per 10^6 cells. Expected PFC response is based on the individual responses of each fraction of the co-culture when cultured alone.

Assay for PFC

At the end of the culture period (6–7 days), cells were harvested and assayed for direct PFC against SRBC by an ultrathin layer haemolysis-in-gel technique as previously described in detail (Fauci & Pratt, 1976a; Fauci & Pratt, 1976b).

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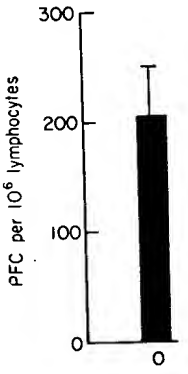

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Blastogenic responses

Blastogenic responses of unirradiated and irradiated lymphocytes to stimulation with the mitogens phytohaemagglutinin (PHA) and PWM were determined in microtitre plates by incorporation of tritiated thymidine as previously described (Fauci 1975).

Statistical analysis

Data were compared by the Student's *t* test or by the paired sample *t* test where indicated.

RESULTS

Effect of irradiation on PFC responses

The effect of directly irradiating the responding cell populations on the PWM-induced PFC responses is shown in Fig. 1. The PFC responses are quite

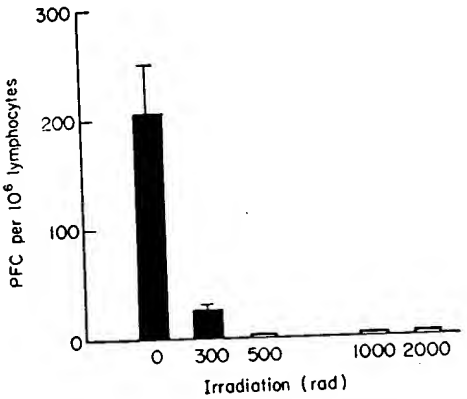

Figure 1. Effect of various doses of irradiation on PWM-induced PFC responses in human peripheral blood mononuclear cells. Unfractionated mononuclear cells were irradiated with from 300 to 2000 rad and subsequently cultured in the presence of PWM. After 6 days, cultures were harvested and assayed for PFC responses against SRBC. Data represent the mean (±SEM) of 10 separate experiments.

sensitive to low doses of irradiation with a marked diminution at 300 rad and a virtually complete suppression of PFC responses at 500 rad or greater. After exposure to 2000 rad, the cell viabilities remained between 75 and 95% after 6 days in culture with only a slight decrease in cell yield. Blastogenic responses to PHA and PWM were also markedly decreased by irradiation as shown in Fig. 2.

It is of interest that despite the fact that PFC responses were markedly decreased with 300 rad and completely suppressed with 500 rad in all individuals tested, quite variable results were seen at very low doses of irradiation. As shown in Table 1, some individuals had an enhancement of PFC responses at very low doses (25 to 100 rad) with the usual suppression at higher doses. However, when mean responses of several individuals at different lower doses of irradiation were examined, there was no dose at which significant enhancement was observed.

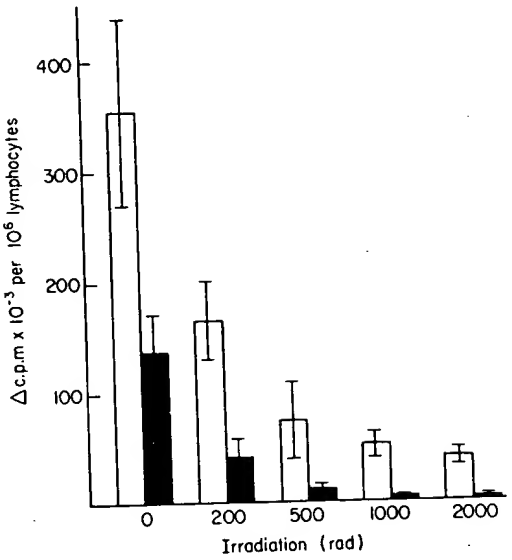

Figure 2. Effect of irradiation on the blastogenic response of human peripheral blood lymphocytes. Mononuclear cell suspensions were irradiated with various doses of irradiation and blastogenic responses were measured following stimulation with PHA (3 day cultures) and PWM (5 day). Blastogenic responses were measured by the incorporation of tritiated thymidine. Δc.p.m. equals the c.p.m. of stimulated cultures minus the c.p.m. of unstimulated cultures. Data represent the mean (±SEM) of 5 separate experiments. PHA, open columns; PWM, filled columns.

Effect of co-culturing unirradiated cells with various fractions of irradiated cells

The effect of co-culturing 1×10^6 unirradiated, unfractionated mononuclear cells with 1×10^6 irradiated autologous T cells is shown in Fig. 3. When unirradiated T cells were co-cultured with autologous unirradiated mononuclear cells there was no significant difference between expected and observed PFC responses. The co-culture of unirradiated cells with autologous T cells which had been

Table 1. Effect of low doses of *in vitro* irradiation on PFC responses in normal human peripheral blood

Subject	No irradiation	PFC per 10^6 lymphocytes* (rad)					
		25	50	100	200	300	500
1	140	725	120	225	90	18	0
2	6	45	195	600	33	0	0
3	156	195	219	99	57	36	0
4	100	54	2	0	0	0	0
5	93	176	30	19	0	48	0
6	49	79	141	114	12	15	2
7	85	27	18	16	1	20	0

* Cell suspensions were irradiated and then cultured in the presence of PWM for 6 days. Viabilities at the end of culture were 85 to 90%.

irradiated with 300 to 500 rad resulted in an obvious trend towards enhancement of responses which fell just short of statistical significance ($P > 0{\cdot}05$) by a paired sample 't'-test. However, the co-culturing of unirradiated mononuclear cells with T cells pre-treated with 1000 to 2000 rad resulted in a marked enhancement of expected responses ($P < 0{\cdot}01$). The same phenomenon was observed although less consistently when mononuclear cells were co-cultured with allogeneic irradiated T cells (Fig. 4).

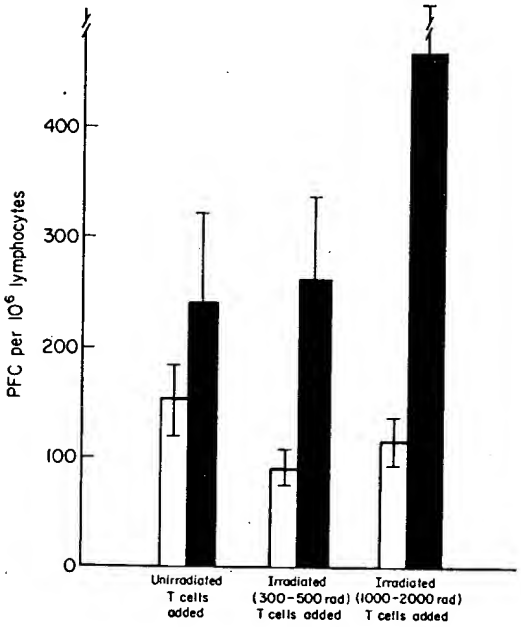

Figure 3. Effect of co-culture of irradiated T cells with autologous unfractionated mononuclear cells. Irradiated and unirradiated cells were co-cultured at a ratio of 1 : 1. Data are given as the comparison of the expected PWM-induced PFC response compared with the observed response. Data represent the mean (±SEM) of seven separate experiments. Expected PFC response, open columns; observed PFC response, filled columns.

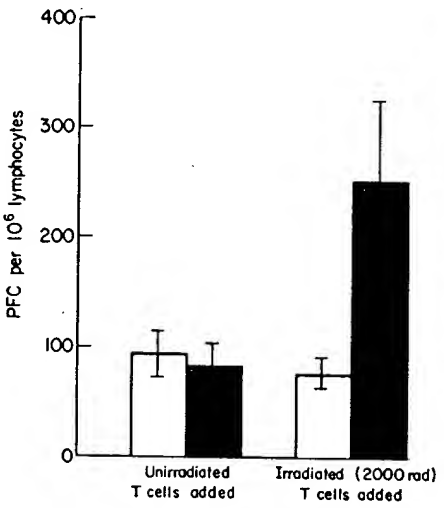

Figure 4. Effect of co-culture of irradiated T cells with allogeneic unfractionated mononuclear cells. Conditions were the same as in Fig. 3. Data represent the mean (±SEM) of nine separate experiments. Expected PFC response, open columns; observed PFC response, filled columns.

The addition of unirradiated allogeneic T cells to PWM stimulated mononuclear cells resulted in no significant differences in mean expected and observed PFC responses ($P > 0{\cdot}2$). However, the co-culture of mononuclear cells with allogeneic T cells irradiated with 1000 to 2000 rad resulted in signifi-

cant enhancement over expected PFC responses ($P<0{\cdot}05$).

The same effect, although less consistently was observed when autologous, irradiated unfractionated mononuclear cells instead of T cells were used, i.e. significant enhancement was observed ($P<0{\cdot}01$). However, it should be pointed out, that as shown above, allogeneic irradiated T cells were essentially as effective as autologous irradiated T cells in enhancing PFC responses of B cells. This was not the case with allogeneic irradiated unfractionated mononuclear cells. They failed to consistently supply the same degree of enhancement as autologous irradiated unfractionated cells.

Irradiated T-cell depleted fractions, either autologous or allogeneic failed completely to enhance PFC responses, and in many cases actually suppressed the responses.

Mitomycin C treatment and heat killing

Similar enhancing effects were observed when T cells were treated with mitomycin C rather than irradiation prior to co-culture. As shown in Table 2, addition of mitomycin C treated T cells to culture resulted in enhanced PFC responses in two of three experiments.

Neither significant enhancement nor suppression of PFC responses was observed when heat killed T cells were co-cultured with PWM stimulated mononuclear cells.

Table 2. Effect of co-culture of unfractionated mononuclear cells with mitomycin C treated T cells on PFC responses

Subject	Cells added	PFC per 10^6 lymphocytes
1	T*	137
	T_{MC}†	260
2	T	167
	T_{MC}	480
3	T	125
	T_{MC}	129
4	T	75
	T_{MC}	375

* T, Fresh untreated autologous T cells preincubated with media alone.

† T_{MC}, Autologous T cells which had been preincubated with 40 μg/ml of mitomycin C for 45 min at 37°, washed four times and then added to culture.

DISCUSSION

The present study demonstrates that functional subpopulations of human peripheral blood lymphoid cells engaged in the PFC response following polyclonal activation are differentially sensitive to various levels of irradiation. The antibody forming cell itself is quite sensitive to relatively low doses of irradiation as 300 rad markedly suppressed PFC responses while 500 rad consistently abolished responses. We have previously shown this PWM-induced PFC system to be T-cell dependent (Fauci *et al.*, 1976) and to operate by a balance between 'helper' and 'suppressor' phenomena (Fauci *et al.*, 1977; Haynes & Fauci, 1977). In this regard, the present data clearly demonstrate that in co-culture experiments T-cell helper effects are radioresistant to as high as 2000 rad. These findings are in agreement with those reported for the radioresistance of helper T cells in PWM-induced intracytocytoplasmic immunoglobulin production (Siegal & Siegal, 1977) as well as for the radioresistance of functional helper T cells identified by the presence of an Fc receptor for IgM and the radiosensitivity of functional suppressor T cells identified by the presence of an Fc receptor for IgG (Moretta *et al.*, 1977).

We have further shown that irradiated T cells can also enhance the PFC responses of allogeneic B cells. This allows a convenient and reproducible method for separately evaluating B-cell function and helper T-cell function in various patient groups by allogeneic co-culture experiments.

Since unfractionated mononuclear cells contain predominantly T cells, one might expect that co-culture of irradiated unfractionated mononuclear cells with unirradiated unfractionated mononuclear cells would also result in enhancement of responses by a selective helper effect. Indeed, we found this to be the case. However, significant and consistent enhancement was only seen with autologous co-cultures of unfractionated mononuclear cells and not with allogeneic co-cultures as was seen with allogeneic irradiated T cells. The reason for this is unclear; however, it is possible that the strong mixed leucocyte reaction which one would expect against the non-T cells in the allogeneic irradiated unfractionated mononuclear cells and not against the

pure T cells in the allogeneic irradiated T cell suspensions masked the helper effect.

It is of interest that in some individuals very low doses of irradiation delivered directly to the responding cell population (Table 1) resulted in enhancement of PFC responses with complete suppression of responses at higher doses of irradiation. Although it seems, in general, that human B cells are more sensitive to irradiation than are suppressor T cells, it is possible that a subpopulation of suppressor cells in some individuals are even more sensitive to irradiation than are B cells, resulting in the occasional enhancement of responses seen at doses of irradiation lower than those which directly suppress B-cell function.

The similarity of this helper effect with mitomycin C treated T cells and not with heat killed T cells strongly suggests that live T cells whose division or mitosis has been blocked are necessary for shifting the balance towards pure helper effect. However, it must be emphasized that irradiation and mitomycin C treatment besides interfering with mitosis can have multiple effects on cells, many of which are not understood. Thus, it is possible but not absolutely certain that the mechanism of supplying 'pure help' with irradiated T cells is by interfering with the mitosis which is necessary for suppressor T cells to fully express their function. The complexity of this issue of mitosis and suppressor cells has recently been emphasized in the mouse model (Tse & Dutton, 1977).

It should also be pointed out that other nonspecific factors may simulate helper effects. The occasional enhancement seen when heat-killed cells or cells irradiated with 10,000 rad resulting in markedly decreased viability (data not shown) were added in co-culture may well be due to the nonspecific adjuvant effects of nucleic acid degradation products released by dead cells (Braun, 1965). However, it is clear that a consistent and reproducible helper effect is seen in co-cultures with normal irradiated (2000 rad) autologous or allogeneic T cells whose viabilities are not significantly decreased.

Thus, this system has potential clinical relevance in the evaluation of functional capabilities of subpopulations of human lymphoid cells involved in antibody production.

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Clinical Response of a Patient With Diffuse Histiocytic Lymphoma to Adoptive Chemoimmunotherapy Using Cyclophosphamide and Alloactivated Haploidentical Lymphocytes

A Case Report and Phase I Trial

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Adoptive chemoimmunotherapy has cured experimentally induced tumors in animals, but its clinical use has been limited. Six patients were treated with refractory neoplasms in a Phase I study with cyclophosphamide (CPM) and alloactivated haploidentical lymphocytes. Patients received an immunosuppressive dose of CPM (800 mg/m²) followed by haploidentical lymphocytes primed *in vitro* with alloantigens in mixed lymphocyte culture (MLC). One week later patients received a second infusion of alloactivated lymphocytes expanded in T-cell growth factor (TCGF). The total number of cells given to each patient progressively increased, with a single patient receiving 35.5×10^9 cells. Transient febrile responses and delayed-type hypersensitivity reactions at the intravenous sites were the only toxicities noted. A complete clinical response lasting 12 weeks was seen in a single patient with diffuse histiocytic lymphoma. Our experience indicates that adoptive chemoimmunotherapy can be given to patients safely and merits further clinical testing.

Cancer 55:552–560, 1985.

CURE OF transplanted, experimentally induced animal tumors can be accomplished by the infusion of appropriately sensitized lymphocytes.[1-4] Animal models have delineated those conditions necessary to obtain a therapeutic response using adoptive immunotherapy. First, adequate numbers of appropriately sensitized lymphocytes must be transferred to animals with a minimal number of residual tumor cells. For example, 2×10^7 immune lymphocytes given to a leukemic mouse when in a temporary remission can be curative.[5] Secondly, one must overcome the tumor-generated suppressor T-cell system, which can affect the antitumor activity of either endogenous or adoptively transferred immune cells.[6] Cyclophosphamide (CPM) given before lymphocyte infusions can eliminate this suppressor population, resulting in an antitumor response not seen with CPM alone.[7,8]

Extrapolation from murine studies indicates that on the basis of body mass, at least 10^{10} immune human lymphocytes would be required to obtain a comparable immunotherapeutic response in patients with cancer. Until recently, *in vitro* culturing and expansion of this number of cells has been virtually impossible, but with the combined use of leukapheresis, bulk culture priming, and T-cell growth factor (TCGF), it is now possible to obtain 10^{10} *in vitro* activated human T-lymphocytes.

Our pilot study[9] investigated the use of alloactivated syngeneic lymphocytes from a healthy donor to his identical twin with relapsed acute myel monocytic leu-

From the Departments of *Human Oncology, †Genetics, ‖Medicine, ¶ Nuclear Medicine, and #Pediatrics, University of Wisconsin Medical School, Madison, Wisconsin.

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TABLE 1. Characteristics of Patients Receiving Adoptive Chemo-Immunotherapy

Patient identification	Sex/age	Diagnosis	Previous therapy	Relationship of donor to patient
A	M/16	Osteogenic sarcoma	Surgical amputation Chemotherapy	Mother
B	M/43	Acute myelomonocytic leukemia	Chemotherapy Radiation therapy	Sister
C	F/35	Recurrent malignant melanoma	Surgery Interferon	Brother
D	M/46	Recurrent malignant melanoma	Surgery Radiation therapy	Son
E	F/50	Recurrent malignant melanoma	Surgery Chemotherapy	Son
F	M/57	Diffuse histiocytic lymphoma Stage IVA	Interferon Chemotherapy	(1) Daughter (2) Son

kemia. Donor lymphocytes were sensitized with alloantigens *in vitro*, expanded in TCGF, and infused intravenously and intraperitoneally. No immediate toxicities were noted following either infusion, but the patient's death from sepsis unrelated to the infusion precluded any extensive evaluation of delayed toxicity or therapeutic benefit.

This report presents the results of a Phase I trial of adoptive chemoimmunotherapy in six patients with cancer unresponsive to conventional treatment.

Patients and Methods

Patient and Donor Selection

Patients in this study had histologically documented malignancies unresponsive to conventional and other experimental therapy. The clinical characteristics of the six patients studied and the relationship of the patients to their donors are listed in Table 1. All were ambulatory and had not received treatment for at least 2 weeks before entry. Criteria for acceptance included no clinical evidence of uncompensated congestive heart failure, stable hematologic (absolute neutrophil count ≥ 1000, platelet count $\geq 100{,}000$), renal, hepatic, and pulmonary ($FEV_1/FVC \geq 50\%$, O_2 saturation $\geq 90\%$) functions. The availability of a healthy histocompatibility antigen (HLA)-haploidentical first-degree relative willing to undergo leukapheresis was also necessary. In those cases where HLA haploidentity could not be based on hereditary factors (*i.e.*, siblings), HLA typing was used. All patients and donors signed informed consent forms approved by the University of Wisconsin Human Subjects Committee.

Lymphocyte Collection

Donor peripheral blood lymphocytes (PBL) were obtained as the buffy coat by-product of platelet pheresis (Badger Red Cross, Madison, WI). Centrifugation at $400 \times g$ separated the buffy coat layer from the platelet-rich plasma. Separation of the lymphocytes from the leukocyte pool was done by Ficoll-Isopaque (Sigma, St. Louis) density gradient followed by washing of the cells with phosphate-buffered saline. The lymphocytes were then resuspended in HS-RPMI (medium RPMI-1640 supplemented with 10% pooled human serum, L-glutamine, penicillin, and streptomycin). This same process was used in obtaining lymphocytes from ten healthy unrelated volunteer platelet donors. These lymphocytes were combined and irradiated (2500 rad) in order to serve as the "pool" of alloantigen-presenting cells.

Generation of T-Cell Growth Factor

Pooled irradiated PBL from six unrelated donors obtained randomly from the Badger Red Cross were cultured at 10^6 cells/ml in the presence of 1% PHA (Difco, Detroit, MI) and 10^5 cells/ml of irradiated, allogeneic, Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell line cells. These were cultured in RPMI medium as modified above, but containing only 1% human serum. Supernatants were collected at 48 hours, filtered, and tested for potency as described previously.[10]

Bulk Culture and TCGF Expansion

Haploidentical donor lymphocytes were initially stimulated in mixed lymphocyte culture (MLC) with irradiated cryopreserved pooled lymphocytes.[11] In 250-ml flasks (Falcon, Oxnard, CA), 25×10^6 haploidentical donor lymphocytes were cultured with 25×10^6 irradiated (2500 rad) pooled stimulating lymphocytes in 50 ml HS-RPMI, modified as described above. Multiple replicate flasks were cultured for each infusion. In the four patients (Patients A, B, C, and D) where cryopreserved autologous tumor cells were available, they were

irradiated (4000 rad) and added as stimulating cells (1×10^6 cells/flask for Patients A and B; 1×10^5 cells/flask for Patient C and 1×10^4 cells/flask for Patient D) to flasks containing 25×10^6 donor lymphocytes and 25×10^6 irradiated pooled stimulating lymphocytes. Following culturing for 6 days at 37°C in 5% CO_2, the lymphocytes were harvested, washed twice, and resuspended in serum-free RPMI. These lymphocytes are designated MLC-activated cells.

In those patients in whom infusion of lymphocytes expanded in TCGF was planned, a portion of the MLC-activated donor cells were continued in culture with HS-RPMI and 25% to 50% TCGF at 10^5 cells/ml. Multiple 250-ml flasks containing 100 ml of this cell suspension were cultured for each patient. After 6 days of culturing, these lymphocytes, designated "TCGF-expanded cells," were harvested, washed, and resuspended in the same manner as the MLC-activated cells. To assure sterility, microbiologic culturing was done selectively before each infusion.

Experimental Design

Before each infusion of MLC cells, patients received intravenous CPM at a dose of 800 mg/m². Twenty-four hours after CPM, MLC-generated lymphocytes were given by intravenous infusion. Each infusion was carried out over 1 hour. The lymphocytes were delivered through intravenous tubing with the interposition of a blood transfusion filter to remove any small clumps of cells. All patients (except Patient A) received TCGF-expanded lymphocytes by intravenous infusion 7 days later. Four weeks after this first cycle, Patients E and F were given additional cycles of CPM, followed by MLC cells and then TCGF-expanded lymphocytes.

Radioactive Indium (^{111}In) Scanning

With each infusion, 1×10^8 cells were cultured with 500 to 600 μCi of indium 111 (^{111}In)-oxine for 30 minutes, washed in sterile normal saline, and mixed with the remainder of lymphocytes to be infused.[12] Viability, as judged by dye exclusion, was greater than 90%. Sequential gamma camera scans were done 1 to 2 hours following the completion of each infusion and at approximately 24 and 48 hours postinfusion. Before the TCGF-expanded lymphocyte infusion, scanning was done to account for background counts from the previous MLC infusion.

Toxicity Evaluation

All patients were hospitalized beginning on the day of the CPM infusion and for 2 days following the cell infusion. Pretreatment physical examination, chest roentgenogram, pulmonary function test, and complete blood count with differential, platelet count, and multiphasic blood analysis served as baseline. Ear oximetry for O_2 saturation was performed before and approximately 4 hours after each cell infusion. Physical examination and clinical and laboratory assessment (hemogram and multiphasic blood analysis) were performed daily during hospitalization and at weekly clinic visits.

In Vitro *Testing*

Donor lymphocytes for each patient were cultured as responding cells for 7 days with media, irradiated autologous lymphocytes ($Donor_x$), pooled allogeneic lymphocytes ($Pool_x$), lymphocytes from a single unrelated individual (X_x), and the patient's lymphocytes ($Patient_x$). Responders and stimulators were both at 1×10^5 cells/0.25 cc well (Flow, McLean, VA) and done in quadruplicate. Proliferation was measured by 6-hour ^{3}HTdR incorporation (New England Nuclear, Boston, MA).

Both MLC-activated and TCGF-expanded cells were tested for cytotoxic function using a 4-hour Chromium 51 (^{51}Cr)-release assay described elsewhere.[13] Targets included lymphocytes from the donor, the patient, an unrelated individual and, when available, the patient's tumor. Cytotoxicity was determined in 0.25-ml microwells with 5×10^3 targets and 1.5×10^5 effectors per well.

Results

Lymphocyte Recovery From Leukapheresis and Cell Culture

The total number of lymphocytes obtained from each donor following leukapheresis and Ficoll purification ranged from 2.8×10^9 to 7.6×10^9 (mean, 5.1×10^9). Percent recovery following alloactivation in MLC ranged from 75% to 333% (mean, 185%). After expansion with TCGF, recovery ranged from 210% to 925% (mean, 587%) of the initial number of cultured cells. Two donors were required for Patient F. Following alloactivation of the patient's daughter's cells, poor expansion with TCGF resulted in insufficient lymphocyte numbers for infusion. The patient's son was subsequently used for two complete cycles with good *in vitro* lymphocyte recovery and function.

Cyclophosphamide Dose and Cell Infusion

All patients received CPM (800 mg/m²) on the first day of each cycle. Twenty-four hours after CPM, MLC-generated lymphocytes were given by intravenous infusion (Table 2).

Seven days after the infusion of alloactivated lymphocytes, TCGF-expanded lymphocytes were infused. The first patient (Patient A) was scheduled to receive only

TABLE 2. Cyclophosphamide Dose and Cell Infusion Numbers for Each Patient

	Patient								
	A	B	C	D	E(1)	E(2)	F(1)	F(2)	F(3)
Dose of cyclophosphamide (Day = 0 of each cycle)	1.3 g	1.8 g	1.5 g	1.4 g	1.3 g	1.3 g	1.5 g	1.5 g	1.5 g
No. of MLC-generated cells infused $\times 10^9$ (Day = 1 of each cycle)	0.5	0.5	2.1	4.7	3.4	4.8	1.0	4.8	11.0
No. of TCGF-expanded lymphocytes infused $\times 10^9$ (Day = 8 of each cycle)	—	0.5	0.8	0.9	2.3	3.0	—	3.7	15.0
Total no. of cells infused $\times 10^9$	0.5	1.0	2.9	5.6		13.5		35.5	

Numbers in parentheses denote cycle in those patients receiving multiple cycles.

MLC: mixed lymphocyte culture; TCGF: T-cell growth factor.

MLC cells. Inadequate cell recovery prevented the infusion of TCGF-expanded cells for Patient cycle F(1). The cell dose of each infusion, as well as the total number of cells infused, progressively increased with each patient. Patient F received a total of 35.5×10^9 cells with single infusions of 11.0×10^9 and 15.0×10^9 MLC-activated and TCGF-expanded cells, respectively, in the third cycle.

Toxicity

The anticipated nausea and vomiting for 24 to 36 hours following the administration of CPM was experienced by all patients. Delayed toxicities from CPM included declines in both leukocyte and platelet counts. In no case did the absolute neutrophil count drop below 500/mm^3, and no infectious complications were seen. Platelet count depression was minimal, and nadirs were never below 100,000/mm^3. Two patients, Patients C and E, complained of minimal hair loss.

No immediate toxicities accompanied the administration of either alloactivated or TCGF-expanded lymphocytes. Blood pressure and pulse, checked frequently during each infusion, showed no appreciable changes. Ear oximetry done before and 4 hours after each of the cell infusions showed a mean decrease of 1.3% in O_2 saturation (range, 0%–5%). No patient reported dyspnea, cough, or chest pain during or after the infusion. Chest roentgenograms of the first two patients 24 hours after infusions showed no evidence of pulmonary infiltrates.

A transient febrile response (temperature (T) > 38°C) was noted following 3 of the 16 cell infusions. This was seen following the MLC-activated cell infusion in Patient C and after both the MLC-activated and TCGF-expanded cell infusions for Patient F(3). The febrile reaction was accompanied by chills in Patient F. Microbiologic culturing of blood and urine were negative, and there was no evidence of hemolytic reaction. In both cases the patients were afebrile within 4 to 6 hours using acetaminophen as the only treatment.

Of the five patients receiving cell infusions through peripheral lines (Patient B had a Hickman catheter), four developed erythema and induration along the venous track of the intravenous injection site 12 to 24 hours after lymphocyte infusions. Gamma camera scanning of the involved area revealed marked ^{111}In-oxine activity corresponding to the venous path and the area of erythema (Fig. 1). This suggested a delayed-type hypersensitivity reaction to the endothelial cells of the vein by the alloactivated haploidentical cells entering in a concentrated suspension at that site. Warm packs were used to treat affected patients, with one patient requiring additional topical steroid cream. No permanent scarring or desquamation was noted.

No evidence of graft *versus* host (GVH)-related complications were noted in any of the patients. A single patient (Patient E) had watery, nonbloody diarrhea as the only symptom 4 hours after the infusion of both MLC-activated and TCGF-expanded cells. Diarrhea resolved within 2 hours without treatment, and subsequent bowel movements were normal. Liver function tests for all patients showed minimal variation with no consistent or significant change.

Distribution of Lymphocytes

Sequential gamma camera scans were done 1 to 2 hours after the completion of each infusion and at approximately 24 and 48 hours postinfusion. Representative scans from a single patient following an MLC-activated cell infusion are shown (Figs. 2A–2D). The mean percent radioactivity (Fig. 3) shows a marked immediate uptake by the lungs of the ^{111}In-oxine. Over a period of

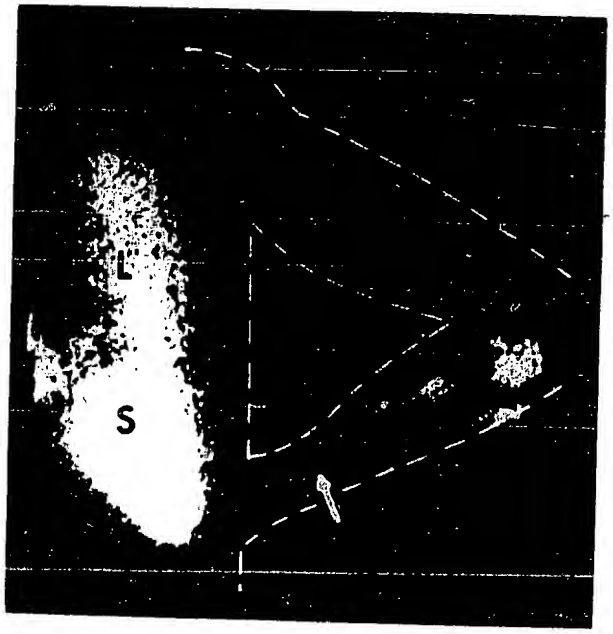
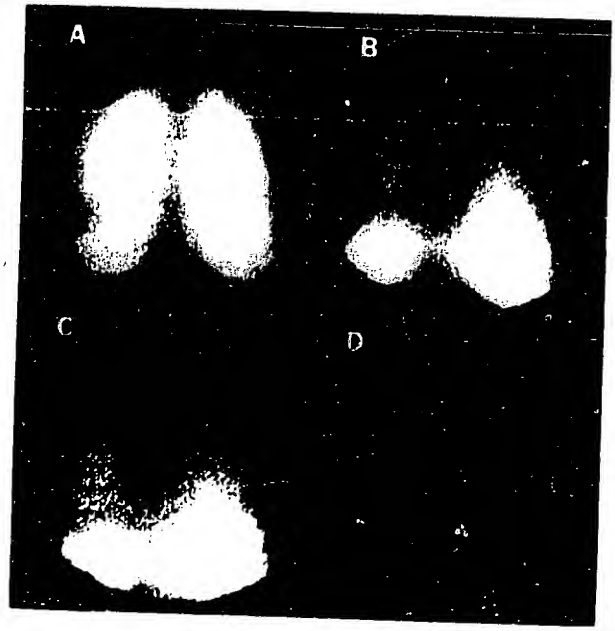


FIG. 1. Delayed hypersensitivity reaction caused by alloactivated lymphocytes. Gamma camera scan at 24 hours of left arm of Patient D showing increased uptake of ^{111}In-oxine-labeled lymphocytes along peripheral vein. The arrow indicates the intravenous site. L: left lung; S: spleen.



FIGS. 2A–2D. Sequential gamma camera scans from Patient D taken following the MLC infusion. Scans A, B, and C are posterior views of thorax and upper abdomen. (A) Scan is 1 hour after infusion and shows the majority of radioactivity localized in both lungs. (B) At 19 hours, the activity in the lungs has decreased, and increased activity is seen in both the liver and spleen. (C) By 46 hours, a further decrease in lung activity is noted. (D) An anterior view of pelvis at 19 hours shows ^{111}In-oxine activity corresponding to the bone marrow.

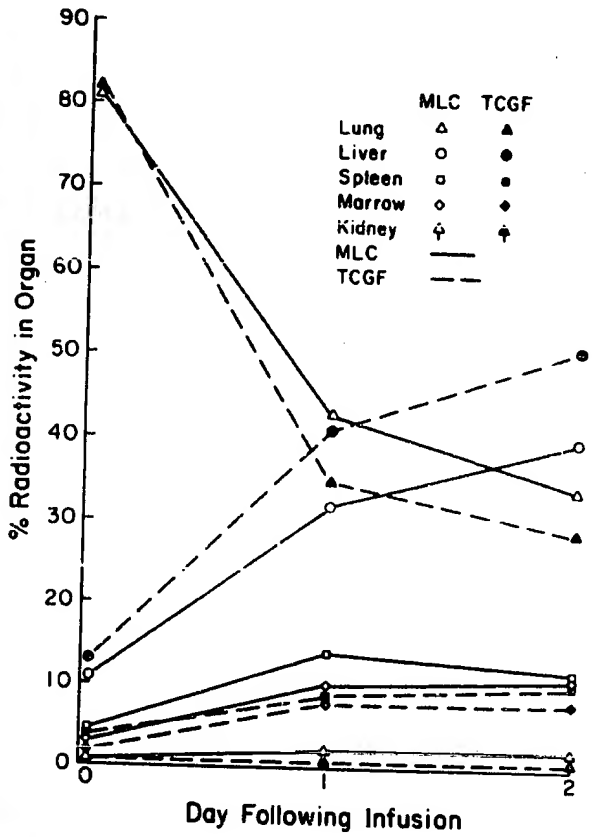


FIG. 3. Summation of ^{111}In-oxine-labeled lymphocyte distribution pattern after MLC and TCGF cell infusions. The percent radioactivity in each organ was determined by initially obtaining counts for a defined area (pixel). The counts/pixel were then multiplied by a volume for each organ standardized for a 70-kg man. Pulmonary counts were obtained for both the right and left lung, and the mean for the two was used. The total counts were obtained by summing the counts/pixel from the lungs, liver, spleen, marrow, and kidneys. Counts from each organ as a percentage of the total counts could then be determined. Calculations for percent radioactivity after TCGF-expanded cell infusion required adjustment for background radioactivity remaining from the previous week's MLC infusion and a decay factor.

24 hours, a decrease in pulmonary activity is accompanied by a progressive rise in hepatic activity. Smaller but easily detectable increases in activity over the 48 hours were seen in the marrow and spleen. No difference in the patterns of distribution between MLC-activated or TCGF-expanded cells could be demonstrated.

In Vitro *Testing*

Primary mixed leukocyte culture (Table 3) demonstrated good proliferative responses by lymphocytes from all but one of the donors tested. Lymphocytes from the donor for Patient D showed poor *in vitro* responses, which may have been an artifact of that day's assay, since a good recovery (260%) of donor lymphocytes followed bulk culture priming.

TABLE 3. Proliferative Responses of Donor Lymphocytes in Primary MLC

Responding cell	Stimulating cell	Donor lymphocytes for patient infusion								
		A	B	C	D	E(1)	E(2)	F(1)	F(2)	F(3)
		cpm ^{3}H-thymidine								
Donor	Media	967	1073	1094	205	139	144	162	2881	1269
Donor	Donor$_a$	720	1385	978	NT	298	196	165	758	NT
Donor	Pool$_a$	39,075	78,997	46,886	166	64,088	100,767	15,645	80,246	23,848
Donor	X$_a$	2118	135,244	490	270	27,739	36,862	25,774	39,655	19,987
Donor	Patient$_a$	21,169	19,568	40,683	NT	23,519	26,267	31,757	12,075	NT

Donor lymphocytes for each patient were cultured as responding cells for 7 days with media, irradiated autologus lymphocytes (Donor$_a$), pooled allogeneic lymphocytes (Pool$_a$), unrelated lymphocytes (X$_a$) or the patient's lymphocytes (Patient$_a$). Responders and stimulators were both at 1×10^5 cells/0.25 ml well and done in quadruplicate. Proliferation was measured by 6-hour ^{3}H-thymidine incorporation.
NT: not tested; MLC: mixed lymphocyte culture.

The cytotoxic activity of the MLC-activated and TCGF-expanded lymphocytes is shown in Table 4. For each infusion, cytotoxic activity by either MLC-activated or TCGF-expanded donor lymphocytes could be demonstrated to either the patient's lymphocytes, the patient's tumor cells, or to lymphocytes from an unrelated person. Low-level cytotoxicity was seen in the MLC-generated lymphocytes for Patient D. This also was most likely the result of technical problems with that day's assay, as lymphocytes from this same culture expanded in growth factor showed a good cytotoxic response.

Clinical Responses

Before entering this Phase I study all patients had received conventional, and in some cases experimental, therapies, with progression of their disease. One patient in this study had a disappearance of all evidence of disease after the first cycle of CPM and MLC-alloactivated haploidentical lymphocytes. Of the remaining five patients, there was no evidence of any therapeutic response, and two patients (Patients A and B) have died of progressive disease.

Case Report

Patient F is a 58-year-old man who presented to his local physician with a superficial swelling over the dorsum of his left wrist in July 1980. After an unsuccessful trial of antibiotics and the development of multiple other skin nodules, a biopsy of the wrist mass was performed. Interpretation of the biopsy at the Armed Forces Institute of Pathology and the University of Wisconsin Hospital and Clinics (UWH) was consistent with lymphoma.

Initial evaluation at UWH revealed multiple nodules involving the back and thigh as well as prominent axillary and cervical adenopathy. Fever, night sweats, and weight loss were denied, and hepatosplenomegaly was not detected on physical examination. Bilateral lower extremity lymphangiogram and bone marrow biopsy specimen were normal. A repeat biopsy of a superficial nodular lesion showed diffuse histiocytic lymphoma (DHL) Stage IVA.

Initial chemotherapy was instituted with 6 monthly cycles of COPA (cyclophosphamide 1100 mg intravenously [IV], vincristine 2 mg IV, prednisone 200 mg orally × 5 days, doxorubicin 90 mg IV). At the conclusion of the sixth cycle (January

TABLE 4. Cytotoxic Responses of Pool Primed Donor Lymphocytes After MLC Activation and TCGF Expansion

Effector	% Cytotoxicity		
	Patient	Tumor	X
Donor Pool$_a$ ± Tu$_a$			
A. *MLC Day = 1	19.1 ± 1.7	0.2 ± 0.3	NT
B. *MLC Day = 1	11.3 ± 1.5	4.4 ± 0.4	
TCGF Day = 8	41.0 ± 1.0	29.5 ± 2.6	35.0 ± 0.6
C. *MLC Day = 1	8.4 ± 2.1	10.9 ± 2.0	NT
TCGF Day = 8	NT	12.4 ± 2.5	NT
D. *MLC Day = 1	0.0 ± 1.3	NT	15.2 ± 1.0
TCGF Day = 8	21.0 ± 9.9	NT	11.6 ± 2.9
E. (1) MLC Day = 1	22.9 ± 2.2	NT	25.6 ± 0.9
TCGF Day = 8	4.7 ± 1.2	NT	25.1 ± 4.1
E. (2) MLC Day = 29	18.6 ± 1.6	NT	25.2 ± 2.6
TCGF Day = 36	26.2 ± 3.9	NT	27.4 ± 1.5
F. (1) MLC Day = 1	NT	NT	38.2 ± 4.3
TCGF Day = 8	NT	NT	NT
(2) MLC Day = 29	3.5 ± 1.2	NT	2.9 ± 0.6
TCGF Day = 36	46.4 ± 8.5	NT	12.0 ± 0.6
(3) MLC Day = 57	18.4 ± 1.2	NT	26.8 ± 3.7
TCGF Day = 64	NT	NT	39.3 ± 2.3

Peripheral blood lymphocytes from each donor were cultured in MLC with irradiated allogeneic pooled lymphocytes (Pool$_a$) and where available (*) irradiated cyropreserved patient tumor cells (Tu$_a$). After 7 days of culturing, the cells were harvested and tested for cytotoxic function using a 4-hour ^{51}Cr-release assay. Targets included lymphocytes from the patient, tumor, and an unrelated individual. MLC-activated lymphocytes were continued in culture with TCGF for an additional 7 days. Cytotoxicity was tested again using the same target cell populations; the effector to target ratio shown is 30:1 in all cases (NT: not tested). The "Day" indicated for MLC and TCGF cells reflects the day of ^{51}Cr-release testing relative to the initial dose of intravenous cyclophosphamide, and in all cases corresponds t the day of infusion of the indicated cell populations. Donors A through F correspond to those in Tables 1 to 3.
MLC: mixed lymphocyte culture; TCGF: T-cell growth factor.

1981) all skin lesions had resolved. Axillary adenopathy was, however, still present. A specimen from a repeat bone marrow biopsy, chest and abdominal roentgenograms, and left axillary node bi psy specimen showed n evidence of lymphoma. Three months after chemotherapy, new skin lesions and cervical adenopathy were noted. Biopsy specimen of a skin nodule showed recurrent DHL. Repeat lymphangiogram, chest roentgenogram, and bone marrow biopsy specimen were normal. Combination chemotherapy was begun with cyclophosphamide (1500 mg IV) vincristine (2 mg IV), and prednisone (200 mg orally), with the disappearance of both skin lesions and cervical adenopathy by the third cycle.

Six weeks after the completion of this second course of chemotherapy, new skin lesions developed that were identical in appearance to those previously documented by biopsy as being lymphoma. Over the next 8-month period, November 1981 through July 1982, the patient received cyclophosphamide (1500 mg IV every 3 weeks × three cycles, 600 mg orally × 5 days every 3 weeks × eight cycles) and prednisone (200 mg orally × 5 days every 3 weeks). Again there was rapid clearing of the lymphomatous skin lesions. On several occasions new skin lesions appeared between cycles; these would resolve following the next cycle of chemotherapy.

From August 1982 through October 1982, multiple new skin lesions and adenopathy appeared despite cyclophosphamide (600 mg orally × 5 days), prednisone, bleomycin (10 mg intramuscularly), and procarbazine (200 mg orally × 10 days) every 3 weeks. The addition of doxorubicin to the above regimen provided an excellent response, but after four cycles of doxorubicin the maximum dose (765 mg) was reached. A radionuclide-determined ejection fraction revealed evidence of cardiac toxicity. Cyclophosphamide, procarbazine, and bleomycin, at the same doses, were continued, but multiple new skin lesions appeared, as did cervical adenopathy.

At the time of this patient's entry to this study, multiple skin lesions were noted on his scalp and face. Also present were painful, visible right cervical and left inguinal adenopathy. The patient received 1500 mg of cyclophosphamide on February 21, 1983 followed in 24 hours by 1.0×10^9 alloactivated lymphocytes (F[1]). Within 7 days, evidence of a tumor response was noted. Before the beginning of the second cycle (4 weeks after the first) there was no adenopathy nor any detectable skin lesions. Two subsequent cycles (F[2] and F[3]) beginning on March 17, 1983 and April 21, 1983 were given. During these 3 months, no adenopathy or skin lesions were detected on weekly examinations. Relapse with multiple skin lesions was noted on May 19, 1983. To determine whether this patient's complete response to the three cycles of adoptive chemoimmunotherapy was solely due to the chemotherapy, cyclophosphamide (1500 mg IV) was given without subsequent cell infusions. Over the next 2 weeks, several of the skin lesions did show evidence of resolution. However, of the lesions noted before treatment, some had progressed and new lesions were noted within 1 week. This was in marked contrast to the complete response seen after cyclophosphamide combined with the immune cells. Two additional monthly cycles of cyclophosphamide alone were given, with no complete resolution of cutaneous lymphoma. A single antecubital lesion grew progressively during these 3 m nths. Finally, the next monthly d se of cyclophosphamide was given with another course of *in vitro* activated lymphocytes from the patient's son (5×10^9 MLC cells on day 1 and 1×10^9 TCGF cells on day 8). By day 8, a complete response of all cutaneous tumor resulted, including resolution of the large antecubital lesion. However, this response lasted only 2 weeks, since new cutaneous lesions were noted on day 22 of this cycle, 3 weeks after the cyclophosphamide with MLC cell infusion, and 2 weeks after the TCGF cell infusion.

Discussion

Appropriately sensitized allogeneic lymphocytes have been shown to have therapeutic activity against established tumors in several animal systems with a demonstrated graft *versus* tumor effect, separate from GVH. Bortin and Truitt[14] were able to improve survival with a minimal increase in GVH in leukemic AKR mice treated with alloimmunized haploidentical lymphocytes. Meredith and OKunewick[15] have recently demonstrated that both major and minor histocompatibility differences are important in obtaining a "graft *versus* leukemia" (GVL) effect and that the magnitude of this GVL effect does not necessarily parallel that of the GVH reaction. The existence of an immune response by HLA-identical lymphocytes to leukemic cells directed at minor histocompatibility antigen has also been shown in humans.[16]

A graft *versus* leukemia effect is suggested in survival analyses of bone marrow transplantation (BMT) patients.[17,18] Following an allogeneic BMT for acute leukemia those patients whose course was complicated by moderate to severe GVH had a lower relapse rate and prolonged survival compared to those patients without GVH. In addition, relapse rates in syngeneic transplants are higher than those seen in allogeneic transplant recipients. Odom *et al.* reported two cases of ALL that relapsed following allogeneic bone marrow transplant but went into remission with the onset of GVH and no other antileukemic therapy.[19] These results suggest that transplanted allogeneic human lymphocytes may have an antineoplastic effect.

The application of adoptive immunotherapy in patients has been limited.[20–23] Thomas *et al.*[20] treated eight patients suffering from acute nonlymphocytic leukemia with their own remission lymphocytes, which were cultured *in vitro* with autologous blasts for 96 hours. No difference in the mean duration of complete response was noted. Nadler and Moore[21,22] paired unrelated patients with malignant melanoma and sensitized each to the other's tumor using subcutaneous melanoma im-

plants. Following graft rejection, leukocytes collected by pheresis were reciprocally infused. Of the 53 evaluable patients, 13 responses were reported. One of the two patients believed to have a complete response remained disease-free for longer than 3 years.

The six patients in this study received alloactivated lymphocytes from a haploidentical relative following immunosuppresive pretreatment with cyclophosphamide. Although HLA-identical donors have a lesser chance of causing GVH, they could share postulated immune response genes that prevent recognition of tumor-associated antigens.[24] Such major histocompatibility complex (MHC)-associated immune response genes have been well-described in animals.[25] This MHC restriction has been shown for T-cell responses to viral, bacterial, and synthetic antigens in human and murine systems, as well as in the recognition of murine tumor cells.[26,27]

Using the methods described here, we were able to generate *in vitro* up to 15.0×10^9 primed lymphocytes for a single infusion. These alloactivated cells were immunologically active *in vitro* and *in vivo*. No significant toxicities, including GVH reactions, were noted following CPM plus MLC-generated or TCGF-expanded lymphocytes despite proven *in vitro* immunologic activity. The absence of a GVH reaction may be due to incomplete suppression of the patient's immune system, which may still have allowed a host-mediated immune rejection of the donor's lymphocytes.

The distribution studies using ^{111}In-oxine labeling of the allogeneic haploidentical lymphocytes expanded in TCGF are in accord with those using small numbers of autologous lymphocytes expanded in TCGF.[28] Following initial uptake in the lungs, these haploidentical cells moved to areas of the reticuloendothelial system (bone marrow, spleen, and liver). Despite the immune reactivity of these cells against patient's normal lymphocytes *in vitro*, the movement of these cells through the pulmonary circulation was not accompanied by any detectable compromise of respiratory function. No difference could be seen between the MLC-generated or TCGF-expanded cells with regard to their distribution. Similar results have been reported for IL-2-dependent T-cell clones in the mouse.[29] Although we did not attempt to show retention of ^{111}In-oxine within the lymphocytes, this has been shown in animal studies.[30]

Two of our patients received more than 10^{10} total cells, which may approximate the magnitude needed to achieve therapeutic responses based on extrapolation from murine models. One of these patients did, in fact, have a complete response to this combined therapy. This complete response was not reproduced by 3 monthly cycles of the same dose of cyclophosphamide used alone, but a transient complete response was then reproduced by a subsequent cycle f cyclophosphamide followed by immune cells.

The biologic mechanism generating this response remains uncertain. Priming with alloantigens has enabled *in vitro* destruction of autologous tumor cells,[11] and preliminary results document a partial clinical response by lymphoblastic lymphoma patients to *in vivo* immunization with pooled allogeneic leukocytes.[31] This suggests that a pool of alloantigens may stimulate the patient's own (or a relative's in the current study) lymphocytes to recognize an antigen on the lymphoma cells that is immunologically cross-reactive with the stimulating alloantigens. Alternatively, alloactivation might nonspecifically activate a separate population of antitumor effector cells, as has been shown *in vitro* with mitogens and lymphokines.[32] In the AKR mouse, alloactivated allogeneic lymphocytes can produce an antitumor response that cannot be generated by syngeneic lymphocytes.[14] This latter may reflect an inability of the tumor-bearing strain to immunologically recognize the tumor antigens.[33] Another possible mechanism would be the immune recognition of genetically controlled major and minor histocompatibility antigens by the allogeneic lymphocytes, with preferential (but not specific) destruction of the neoplastic cells.[24]

Adoptive chemoimmunotherapy using CPM and more than 10^{10} alloactivated haploidentical lymphocytes activated in MLC and expanded in TCGF has been given to these patients without significant toxicity. In addition, evidence presented here suggests that a therapeutic effect has been seen in a patient with a non-Hodgkin's lymphoma. Treatment of more patients with similar tumors is needed to verify this finding; the *in vitro* dissection of the mechanism underlying this effect may provide a more specific clinical approach.

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IN VIVO ANALYSIS OF ALLOGENEIC LYMPHOCYTE INTERACTION: ACTIVATION OF SUPPRESSOR T CELLS BY AN *I-J*-RESTRICTED ALLOGENEIC EFFECT FACTOR (AEF)[1]

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The preceding paper detailed the production and fractionation of a T cell-derived I-J-specific allogeneic effect factor (AEF) and analyzed its ability to provide help in a T cell-depleted, *in vitro* primary anti-sheep erythrocyte response. The identical AEF fractions were examined in this study for their ability to elicit suppression in a delayed-type hypersensitivity assay. Previous reports showed that low or suboptimal doses of antigen, presented i.v. on a cell surface, induce a precursor or primed set f suppressor T cells (preTs). These cells manifested antigen-specific suppression only in the presence of a T cell-mediated I-J-specific allogeneic effect induced *in vivo* against the preTs. The experiments reported here examined the ability of alloactivated T cell-derived I-J-specific AEF components to replace the *In vivo* I-J allogeneic effect. The results show that certain AEF components can indeed provide the signal(s) necessary for activation of suppression. Size and charge separation of the crude AEF preparation revealed several components, some of which could independently serve as appropriate inductive signals. One of these components proved to be biochemically identical to interleukin 2 (IL 2) and accounted for some of the genetically unrestricted AEF activity observed; other higher m.w. molecules also possessed unrestricted activity. Another component provided the requisite activational signals and this 68,000-dalton, pI 5.6 molecule(s) was I-J restricted. These findings are discussed in terms of models of lymphocyte subset interactions and activation.

The mixed lymphocyte reaction (MLR) has been used to study the interaction of primed and unprimed lymphocytes with foreign major histocompatibility complex (MHC)[3] determinants. The vigorous response of the MLR is currently taken as a reflection of the cross-reactivity between "antigens plus self" and allogeneic MHC determinants (1). From this standpoint, the MLR is useful because it is a strong, easily reproducible response and may provide an approach to understanding the nature of the T cell receptor and its interaction with antigen and MHC determinants. In addition, a number of investigators induced MLR to examine the products of the stimulated T cells and to gain more insight into T cell interaction structures (2–4). It is clear from these studies that a variety of soluble products are generated by allogeneic cell interactions and that the idiosyncracies of individual assay procedures may bias the interpretation as to the specificity or restricted nature of the products and their effects on B or T cells. One of us (T.L.D.) has extensively pursued this problem by inducing I-A, I-J, and *Mls*-restricted allogeneic effects *in vivo* and the homologous MLR *in vitro* and by examining whole and fractionated culture supernatants for helper activity on primed and unprimed B cell cultures depleted of T cells (5–8). These studies have provided evidence for both genetically restricted and unrestricted T cell-derived products that help B cell responses, via a direct interaction with antigen-presenting cells (APC) (10). Similar data were generated by Andersson and Melchers (9) using cloned helper T cells as a source of factors in a syngeneic system. The fact that molecules generated by both allogeneic and syngeneic systems will yield experimental data that are compatible with and support one another is a confirmation of the belief that the study of these allogeneic interactions and molecules is likely to be relevant to normal immunologic processes.

The preceding paper (10) showed that the components of an *in vitro* T cell-derived I-J-specific allogeneic effect factor (AEF) could provide both genetically restricted and unrestricted help in a T cell-depleted, *in vitro* primary anti-sheep erythrocyte (SRBC) plaque forming cell (PFC) assay. These same AEF fractions were tested in this report for their ability to activate suppresssion in delayed-type hypersensitivity (DTH) and contact sensitivity (CS) systems in which suppressor T cell precursors (preTs) required a second signal in addition to antigen to display antigen-specific suppression. We showed previously (11, 12) that i.v. injection of syngeneic spleen cells conjugated with high concentrations of hapten induced Ts for DTH and CS whereas i.v. injection of cells chemically coupled with low epitope densities of hapten generated inactive preTs. To become fully activated, the preTs had to be subjected to an I-J-specific allogeneic effect *in vivo*. Allogeneic effects directed at H-2K or H-2D, I-A, I-B, *Mls*, H-Y, or background alloantigens were incapable of activating or otherwise inducing the preTs. In addition, parental and F_1 combinations showed that the preTs was the *recipient* of the allogeneic effect; I-J allogeneic effects of the incorrect directionality were also ineffective signals. The preTs was found to be Thy-1^+; Lyt-1^+2^-; I-J^+; and, in a DTH system controlled by idiotypic regulations, idiotype$^+$. The cell responsible for providing the allogeneic signal *in vivo* was Thy-1^+ and Lyt-1^+ 2^-. This experimental system therefore provided the unique opportunity to investigate the *in vivo* effects on Ts of a number of alloactivated T cell-derived molecules defined by their *in vitro* effects on APC and/or B cells. It will be shown that such molecules are active both *in vitro* and

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[3] Abbreviations used: ABA, azobenzenearsonate; AEF, allogeneic effect factor; APC, antigen-presenting cell; CS, contact sensitivity; DTH, delayed-type hypersensitivity; IL 2, interleukin 2; MHC, major histocompatibility complex; preTs, suppressor T cell precursor.

in vivo, and that their activities are conferred by different modes of action.

MATERIALS AND METHODS

Mice. B10.A (3R) mice were maintained in our animal facilities. All other strains were purchased from the Jackson Laboratory, Bar Harbor, ME.

Allogeneic effect factor (AEF). The AEF was produced as described in detail in the accompanying paper (10). A variety of AEF were generated, including a B10.A 3R anti-B10.A 5R combination and B10.A 5R anti-B10.A 3R combination. The AEF was concentrated and fractionated by sizing and chromatofocusing to investigate the possible presence of distinct activities. Briefly, anti-I-J allogeneic effect factor was fractionated at 4°C over an Ultrogel ACA54 column (Fisher Scientific Co., Toronto, Ontario) with approximate m.w. assigned to the eluted fractions. Selected fractions were pooled in 8-ml volumes and were further resolved by chromatofocusing at 4°C on a 1 x 20 cm column of PBE 94 gel (Pharmacia Fine Chemicals, Dorval, Quebec) by using 0.025 M imidazole-HCl as start buffer and polybuffer 74-HCl, pH 4.0 as eluent. A variety of batches of AEF were prepared. Each batch activity was verified in the suppression assays indicated below and also in the assays described in the accompanying manuscript (10).

Experimental design. This has been described in detail (11, 12). Briefly, to generate preTs, mice were i.v. injected with 5×10^7 syngeneic spleen cells chemically coupled with 0.01 mM trinitrophenyl (TNP) or 1.26 mM azobenzenearsonate (ABA). One week later, these animals were sacrificed and their spleens were used as a source of antigen-specific preTs. Fifty million preTs were transferred i.v. into syngeneic recipients that were also immunized the day of transfer. Immunization to TNP was accomplished with 100 μl of 7% picryl chloride in 4:1 acetone:olive oil applied to the shaved abdomen. Immunization to ABA was accomplished with 3×10^7 12.6 mM ABA-coupled syngeneic spleen cells injected subcutaneously into two sites on the dorsal flank. Five days after immunization, mice were challenged on the ears for CS to TNP or in the footpads for DTH to ABA. Twenty-four hours after challenge, ear or footpad swelling was quantitated with an engineer's micrometer and the results were expressed as the change in thickness of the ears (before *vs* after challenge) or the footpads (the challenged *vs* the unchallenged footpad). There were four to five mice per group and statistical comparisons were made with the Student two-tailed *t*-test.

To activate preTs, an allogeneic effect was induced *in vivo* by cotransferring 3×10^7 normal, allogeneic spleen cells i.v. with the preTs into recipients syngeneic to the preTs. Alternatively, the preTs were incubated with whole or fractionated AEF supernatants (10^8 cells/ml, 37°C, 30 min with agitation) at the AEF dilutions designated and then, without washing, were injected i.v. into syngeneic recipients.

RESULTS

In vivo activity of unfractionated AEF. PreTs were induced as stated in *Materials and Methods* by i.v. injecting syngeneic cells coupled with low doses of hapten into mice. The animals were sacrificed 1 wk later and their spleens were used as a source of preTs to be transferred into syngeneic recipients. To produce an allogeneic effect and the necessary allogeneic signals to induce suppression, normal allogeneic cells were cotransferred along with the preTs. As shown in Figure 1 (line I–IV), preTs plus allogeneic cells suppressed TNP CS whereas neither cell population alone had any effect. The simplest explanation, as discussed previously (11, 12), is that the allogeneically derived signals are involved in activating or augmenting the activity of the preTs and causing suppression. This suppression was also shown to be quite specific in both its inductive and effector phases (11, 12). To test for the activity of AEF derived from the B10.A (3R) stimulated by B10.A (5R) lymphoid cells in replacing the allogeneic effect, preTs were incubated with varying dilutions of unfractionated AEF for 30 min at 37°C and then were injected, without washing, into syngeneic recipients. Figure 1*A* shows that preTs generated by 0.01 mM TNP-coupled spleen cells were able to suppress TNP-specific CS when preincubated with an I-J AEF. Neither preTs by themselves nor normal spleen cells preincubated with AEF were able to provide any significant degree of suppression. Figure 1*B* shows that preTs specific for ABA were also activated by exposure to AEF. Furthermore, the suppression mediated by this experimental protocol was antigen-specific; DNP-specific CS generated in the same animals was

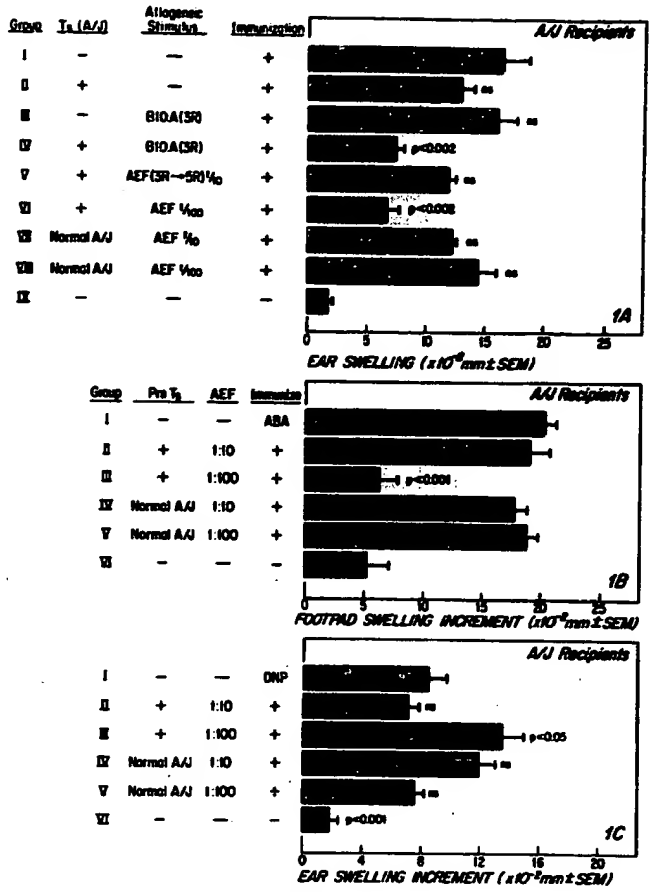

Figure 1. AEF can provide an activational signal. PreTs were generated in A/J mice (5×10^7 0.01 mM TNP (*A*) or 1.26 mM ABA- (*B*) coupled spleen cells, i.v., 1 wk previously) and 5×10^7 were transferred to syngeneic recipients alone, with 3×10^7 B10.A(3R) allogeneic cells (*A*), or after incubation with a 3R anti-5R AEF (30 min, 37°C, 10^8 cells/ml) at the designated dilutions. Recipients were immunized with TNP (*A*), ABA (*B*), or dinitrophenyl on the day of transfer and were challenged 5 days later with the homologous hapten to elicit CS or DTH. Normal A/J refers to the transfer of normal spleen cells, instead of preTs, incubated in AEF.

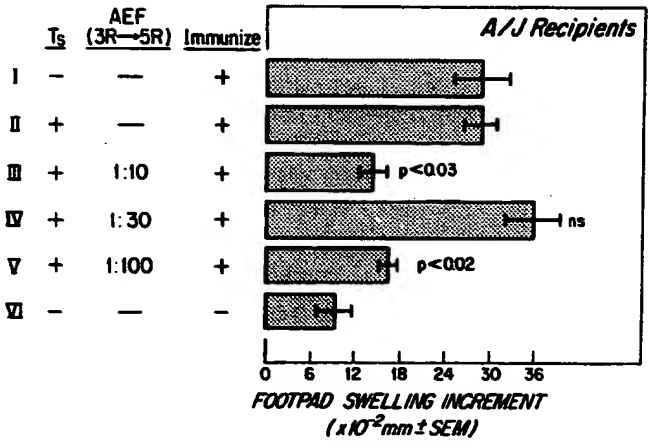

Figure 2. Titration of AEF. PreTs were generated in A/J mice, incubated with varying dilutions of a 3R anti-5R AEF, and injected into syngeneic recipients that were immunized the same day and were challenged 5 days later with ABA.

not affected. In fact, in some cases (Fig. 1*C*, group III), DNP-specific reactivity was enhanced rather than suppressed.

Figure 1 also shows that the AEF was more effective at higher (1/100) than at lower dilutions (1/10). This is reinforced by the results presented in Figure 2, which demonstrate that the particular preparation of AEF can have widely varying activities depending on the concentration at which it is employed. Another batch of AEF was used in this study. This variability of activity

has been noted for both I-J and I-A AEF in the PFC assay (5–10). The source of this apparent variability is at least twofold. First, the spleen cell population containing the preTs is obviously a heterogeneous mixture of partially or completely primed and unprimed cells that belong to various effector or suppressor subpopulations. It is conceivable that discrete signals varied in a quantitative manner could elicit quite different effects, as measured by an *in vivo* assay. Secondly, there is likely to be more than one component in the AEF preparation active in this assay (5, 10). A competitive and/or synergistic effect of various components at different concentrations could result in the observed variability.

Fractionation of AEF into distinct functional components. Further support for the notion that the I-J AEF contained several distinct components is shown in Figure 3. PreTs were generated by A/J mice, incubated with 3R anti-5R (anti-I-J^k) or 5R anti-3R (anti-I-J^b), AEF, and injected into syngeneic recipients. Both AEF preparations were able to suppress, albeit at different concentrations, and hence appeared to be H-2-unrestricted. This is in apparent contrast to results in the previous report (10), which showed this same I-J AEF to be H-2-restricted in the PFC assay. The unrestricted activity, however, is presumably attributable to the AEF interleukin 2 (IL 2) component that is optimally active in the 1/10 to 1/100 dilution range of unfractionated AEF (5, 10). The gentically restricted AEF activity may be observed only in the 1/1000 to 1/10,000 dilution range and is likely conferred by another component (5, 6, 10).

AEF was therefore fractionated by gel filtration on Ultrogel ACA 54 and then by chromatofocusing (10) to investigate the possible presence of distinct *in vivo* activities in the unrestricted and restricted components. Table I shows the results obtained with various fractions of an anti-I-J^k AEF separated on ACA 54. (See Figure 1 of Reference 10 for chromatogram). It is apparent that there are at least two distinct activities, one conferred by a 90 to 95K m.w. fraction and the other by a 30 to 40K m.w. fraction. Similar data were obtained with the low m.w. (12 to 40K) and high m.w. (70 to 110K) components of other AEF preparations (see Tables II and III).

It was shown previously (5, 10) that the low m.w. activity of I-A and I-J AEF contained IL 2 by the criteria of molecular size, isoelectric point, and T cell stimulatory activity. The I-J AEF analyzed here was subjected to similar analyses. A 3R anti-5R AEF was passed through an ACA 54 column and the fractions corresponding to 30 to 35K were pooled, concentrated and chromatofocused. Fractions eluted at different pH were then tested for their ability t provide a second signal to preTs. Table IV shows that the pH 4.0–5.0 fractions contained potent suppressive activity. The other fractions elicited lower levels of suppression comparable to that achieved by the transfer of preTs alone (data not shown). Therefore a 30 t 35K, pI 4.0–5.0 molecule(s) is able to activate preTs. Such physicochemical criteria mak it likely that this component is IL 2. Furthermore, this fractionated material was able to influence proliferating and cytotoxic T cells *in vitro* in accordance with established IL 2 activities (5). Additional experiments (not shown) demonstrated that the fractionated AEF did not suppress when administered alone. Because IL 2 is known to be neither allogeneically nor even xenogeneically restricted (13), the presence of IL 2 in the I-J AEF and its activity in the suppressor assay when admixed directly with preTs can account for the apparent nonrestriction observed in Figure 3. Further studies on this IL 2-like component were deferred and experiments were concentrated on the functional activity of other fractions, as indicated below.

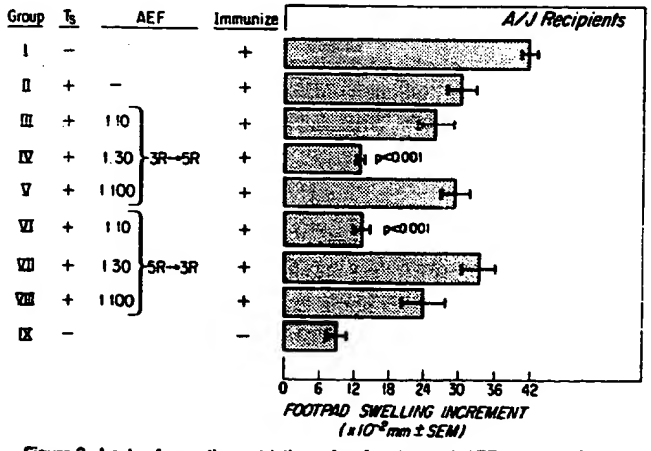

Figure 3. Lack of genetic restriction of unfractionated AEF. A/J preTs were incubated with varying dilutions of a 3R anti-5R (III-V) or a 5R anti-3R (VI-VIII) AEF and were injected into syngeneic recipients.

The I-J AEF was also shown to possess a 68K, pI 5.6 fraction, designated T_H-II, which provided I-J^k-restricted activity in the PFC assay (10). As shown in Tables I and III, however, no suppressive activity was demonstrated in the 50 to 70K range of the anti-I-J^k AEF. Nonetheless, the identical m.w. fractions did possess potent, I-J^k-restricted activity in the *in vitro* PFC assay. To confirm this restricted component was inactive in the DTH suppressor assay, the 50 to 70K m.w. component was chromatofocused and the various fractions of different pH were tested. *Unexpectedly*, but quite interestingly, activity was revealed in only the pH 5.5–6.0 fraction (Table V). Therefore, the *I-J*^{k}-restricted T_H-II component that operates at the level of APC in the *in vitro* helper assay was active at the level of preTs in the *in vivo* suppressor assay. The inability to find Ts activity in fractions separated only by size may be due either to the presence of an inhibitor that is removed by isoelectric focusing or could merely be the result of the semiquantitative estimates of AEF concentration.

In Table VI are the results of another experiment in which both the 50 to 70K, pI 5.5–6.0 (Table V) and 70 to 110K (Table II) fractions of the anti-I-J^k AEF were tested in *I-J*^{k} syngeneic (A/J) and *I-J*^{b} allogeneic (A.BY) recipients. As before, both fractions suppressed the A/J strain; however, only the 70 to 110K component activated A.BY preTs cells. Therefore, this latter fraction was unrestricted in its activity whereas the 50 to 70K, pI 5.5–6.0 component was H-2-restricted, similar to its activity in the PFC assay. As a further control on the genetic specificity of this fraction, it was tested in A/J mice with an anti-I-A^k AEF (5). The I-J AEF provided 49% suppression of the response, compared to the I-A AEF, which induced 0% suppression. Therefore, it is necessary that the AEF components be restricted not only to the proper haplotype but also to the proper I subregion (i.e., *I-J*) to express activity in the DTH suppressor assay. It is likely that the *I-J*^{k}-restricted 68K m.w., pI 5.6, T_H-II component identified in the accompanying paper confers the activity observed here in the DTH response.

DISCUSSION

We focused this analysis of the products of an I-J-specific AEF upon a system in which the activation of Ts seemed dependent on two signals: antigen and an I-J-specific allogeneic effect. Our earlier reports (11, 12) showed that low or suboptimal doses of hapten induced a set of antigen-specific Ts that would suppress only in the presence of an I-J allogeneic effect directed against these cells *in vivo*. This directionality of the allogeneic effect argued for a simple two-signal hypothesis. Indeed, subsequent

TABLE I

Size fractionation of AEF[a]

	PreTs	AEF 3R anti-5R	Immunize	Footpad Swelling Increment (× 10^{-2} mm ± SEM)	Percent Suppression
I	–	—	+	29.0 ± 4.5	
II	+	1:10	+	20.5 ± 3.1	
III	+	1:30 unfractionated	+	25.75 ± 3.6	
IV	+	1:100	+	*10.5* ± *0.5* $p < 0.01$	84
V	+	105K	+	25.0 ± 2.2	
VI	+	90–95K ACA fractions @ 1:15	+	*12.0* ± *0.9* $p = 0.01$	77
VII	+	65K	+	24.75 ± 1.4	
VIII	+	30–40K	+	*13.0* ± *2.6* $p < 0.04$	73
IX	+	—	+	28.0 ± 1.7	
	–	—	–	7.0 ± 0.9	

[a] Unfractionated 3R anti-5R AEF or fractions from an Ultrogel ACA-54 column were incubated with A/J preTs (30 min, 37°C) that were then injected into syngeneic recipients. Recipients were immunized the day of transfer and were challenged 5 days later with ABA.

TABLE II

Size fractionation of AEF: estimates of m.w. of tested fractions[a]

	PreTs	3R anti-5R ACA Fractions @ 1:5	Immunize	Footpad Swelling Increment (× 10^{-2} mm ± SEM)	Percent Suppression
I	–	—	+	27.75 ± 3.4	
II	+	A (>200K)	+	25.7 ± 6.4	
III	+	B (150–200K)	+	29.0 ± 4.0	
IV	+	C (110–150K)	+	32.2 ± 1.3	
V	+	D (70–110K)	+	*12.7* ± *1.8* $p < 0.02$	82
VI	–	—	–	9.5 ± 2.3	

[a] The initial fractions (A–D) of a 3R anti-5R AEF passed over an ACA-54 column were tested for activity.

TABLE III

Size fractionation of AEF: low m.w. cuts[a]

	PreTs	3R anti-5R ACA Fractions @ 1:15	Immunize	Footpad Swelling Increment (× 10^{-2} mm ± SEM)	Percent Suppression
I	–	—	+	22.5 ± 1.9	
II	+	40–70K	+	18.7 ± 0.7	
III	+	20–40K	+	7.7 ± 1.2 $p < 0.01$	100
IV	+	12.5–20K	+	8.3 ± 2.6 $p < 0.01$	98
V	+	10K	+	15.3 ± 3.8	
VI	–	—	+	19.75 ± 0.9	
VII	–	—	–	8.0 ± 4.4	

[a] Low m.w. cuts of the same AEF preparation as in Table II.

TABLE IV

Chromatofocusing of AEF: 30,000–35,000 m.w. fraction[a]

	PreTs	AEF pH Fractions from Chromatofocusing of 30–35K Cut @ 1:3	Immunize	Footpad Swelling Increment (× 10^{-2} mm ± SEM)	Percent Suppression
I	–	—	+	36.0 ± 2.0	
II	+	3.7–4.5	+	*16.3* ± *4.1* $p < 0.005$	69
III	+	4.5–5.0	+	*18.75* ± *1.0* $p < 0.001$	60
IV	+	5.0–5.5	+	27.75 ± 1.8	29
V	+	5.5–6.0	+	25.75 ± 3.4	36
VI	+	6.0–6.5	+	27.0 ± 1.5 $0.01 < p < 0.05$	31
VII	+	6.5–7.0	+	27.75 ± 2.2	29
VIII	–	—	–	7.24 ± 0.5	

[a] A 3R anti-5R AEF (same as in Table III) was chromatographed on ACA-54, and the 30,000 to 35,000 m.w. fraction was then subjected to chromatofocusing. Fractions that differed by about 0.5 pH units were tested for their ability to induce A/J preTs.

TABLE V

Chromatofocusing of AEF: 50,000 to 70,000 m.w. fraction[a]

	PreTs	AEF 3R anti-5R pH Fractions from Chromatofocusing of 40–70K @ 1:3	Immunize	Footpad Swelling Increment (× 10^{-2} mm ± SEM)	Percent Suppression
I	–	—	+	20.25 ± 2.25	
II	+	3.7–4.5	+	15.5 ± 4.5	
III	+	4.5–5.0	+	21.0 ± 3.4	
IV	+	5.0–5.5	+	16.5 ± 1.6	
V	+	5.5–6.0	+	*10.0* ± *1.3* $p = 0.0075$	100
VI	+	6.0–6.5	+	19.5 ± 1.3	
VII	+	6.5–7.0	+	16.5 ± 1.9	
VIII	–	—	–	11.0 ± 0	

[a] A 3R anti-5R (same in Table III) was fractionated by an ACA-54 column and the 50,000 to 70,000-dalton cut was subjected to isoelectric focusing and fractions representing 0.5 pH units were tested on A/J preTs.

work (Reference 14; J. S. Bromberg and M. I. Greene, manuscript in preparation) extends the notion that two signals can induce Ts. The first (antigen) interacts with a clonally distributed receptor and the second is an activational signal that interacts with a nonclonal receptor on the cell surface. The results in this report show that the allogeneically derived second signal required for the complete activation of suppression may be derived from either allogeneic cells (previously shown to be Lyt-1^+2^-) or a

TABLE VI

Genetic restrictions of AEF activity[a]

PreTs	AEF 3R anti-5R	Immunize	Footpad Swelling Increment ($\times 10^{-3}$ mm ± SEM)	Percent Suppression
A/J I+	Inactive fraction (1:3)	+	36.5 ± 3.3	
II+	40–70K, pI 5.5–6.0 (1:4)	+	*18.5* ± 3.6 $p = 0.01$	58
III+	70–110K (1:5)	+	*17.3* ± 3.2 $p < 0.01$	62
IV−	—	−	5.5 ± 1.0	
A.By V+	Inactive fraction	+	29.3 ± 3.2	
VI+	40–70K, pI 5.5–6.0	+	32.0 ± 2.2	
VII+	70–110K	+	*21.3* ± 4.2 $p = 0.05$	43
VIII−	—		7.25 ± 1.9	

[a] The 50,000 to 70,000 dalton, pI 5.5–6.0 fraction (Table V), 70,000 to 110,000 dalton fraction (Table II), and the inactive fraction A (Table II) of a 3R anti-5R AEF (anti-$I\text{-}J^b$) were tested on A/J ($I\text{-}J^b$) and A.BY ($I\text{-}J^b$) preTs that were transferred to syngeneic recipients after incubation.

culture supernatant AEF (also produced by Lyt-1^+2^- cells). The AEF was fractionated and shown to contain several distinct components that *independently* were able to provide an adequate second signal. One component was 30 to 35K, pI 4.0–5.0, and was most likely IL 2. A second was 90K and H-2-unrestricted in its activity. The third component was 50 to 70K, pI 5.5–6.0 and H-2-restricted in its activity to preTs of stimulator type. The relationship of these activities to one another is not known. For example, the very high m.w. component(s) could be aggregates of smaller species. In addition, it is apparent (Table III) that there is activity in components ≤ 20K that may represent distinct molecules or degradation productions of larger ones.

The ability of purified IL 2 to activate suppression in this system was unexpected because allogeneic effects at K, D, I-A, and *Mls* generate large amounts of IL 2 (13) yet were ineffective at inducing suppression (11) *in vivo*. The lack of activity of IL 2, when induced *in vivo*, implies it does not primarily mediate the second signal function. Therefore its ability to act when presented *in vitro* to the splenic suppressor cell population argues that the *in vitro* protocol represents an anomolous sequestration of factor or cell populations that does not occur *in vivo*. For example, the concentration of IL 2 admixed *in vitro* with preTs may be higher than that achieved *in vivo*. Alternatively, a whole array of effector and suppressor cells could be exposed to IL 2 *in vivo*, whereas a population relatively more enriched in suppressors would be exposed *in vitro*. Hardt *et al.* (15) recently showed that allogeneic Lyt-2 cells induce a serum inhibitor of IL 2 *in vivo*. Therefore it may not be surprising that IL 2 does not seem to function in our protocols, which employ an *in vivo* allogeneic effect, whereas it does function as a component of AEF. This further implies that in "normal" physiologic lymphocyte interactions, IL 2 may be a growth hormone for Ts. This notion should be contrasted with recent reports (16, 17) in which it is shown that some suppressor cells act by compromising the activity or production of IL 2.

The ability of an *I-J*-restricted AEF component to activate suppression is consistent with the *in vivo* requirement for an I-J-specific allogeneic effect and the $I\text{-}J^+$ surface phenotype of the preTs (12). The fact that an anti-$I\text{-}A^k$ AEF did not similarly induce suppression could imply that the *I-J*-restricted AEF molecule(s) interacted directly with the preTs or another $I\text{-}J^+$ T cell rather than with an $I\text{-}A^+$, $I\text{-}J^+$ APC as demonstrated for the effect of this same AEF fraction in the PFC assay (10). This reasoning opens up the question as to the site of action of the various AEF functions investigated in this study. The experimental design does not permit one to conclude definitively whether the molecules were acting at the level of the preTs, APC, or some other cell type. As a corollary, the ability of biochemically distinct activities to *independently* provide a second signal may mean that a multitude of these signals can activate preTs or that each component is acting at a separate locus in a linear cellular pathway. If the latter case were true, then it may be expected that the more specific, genetically restricted molecules act early in the pathway to result eventually in the production of the less specific factors. How preTs, APC, and other T cells could fit into such a pathway is presently unclear.

The striking parallel of AEF activities in the *in vitro* helper and the *in vivo* DTH suppressor assays provides a model for normal lymphoid interactions in the induction of Ts. The recognition of allogeneic I-A by T cells is taken as a reflection of the cross-reactivity between self I-A plus antigen (or altered self) and allogeneic I-A. The production of an antigen-specific, I-A-restricted factor(s) by helper T cells in the syngeneic environment would be mirrored by an allogeneically I-A-restricted factor(s) resulting from allogeneic interactions (8, 9). It is postulated that APC I-A seems to stimulate the T cell production of factor(s) whereas B cell I-A and antigen bound to cell surface immunoglobulin serve as the targets for such factor(s), although it cannot be excluded that APC could also be the targets of such products, especially those resulting from syngeneic or allogeneic I-J-restricted interactions (6, 8, 18). By analogy, self I-J plus antigen or allogeneic I-J would stimulate a helper-type (Lyt-1) T cell to produce a factor(s) specific for the stimulating moieties. The target of such factor(s) could be either APC or $I\text{-}J^+$, idiotype$^+$ (i.e., antigen binding) preTs. Such models rely on the differential distribution of Ia antigens to preserve symmetry of cellular interactions in the immune response and to maintain a separation of functional pathways while minimizing the number of distinct tasks a lymphocyte subset actually needs to perform.

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Alloactivated long-term cultured human T lymphocytes express both HLA-DR and SB antigens but lack lymphocyte stimulation capacity*

Cell populations obtained from mixed leukocyte cultures of 6- or 10-day duration were found specifically to restimulate primed lymphocytes detecting HLA-linked SB as well as HLA-D-associated antigens. After expansion *in vitro* (9–75 days) with medium containing interleukin 2, the cultured cells expressed the T lymphocyte markers detected in indirect immunofluorescence by monoclonal antibodies Lyt-3, OKT3, OKT4, OKT8, and had high levels of HLA-DR antigens. In addition, they were shown in cell-mediated lymphocytotoxicity specifically to express SB antigens of the donor B cell type. Despite their positivity for DR and SB antigens, such cultured T cells failed to restimulate either SB- or D-specific secondary lymphocyte proliferation. Homogeneous cloned populations of cultured T cells also lacked lymphocyte stimulation capacity. In contrast, B cell lines, which also expressed DR and SB antigens, were potent stimulators of both SB- or D-directed proliferation. These data show that the activated T lymphocytes which express both HLA-DR and SB antigens are by themselves unable to stimulate lymphocyte proliferation.

1 Introduction

Normal resting peripheral blood T lymphocytes express very little, if any, detectable HLA-DR-antigen. However, the majority of T cells stimulated by soluble antigen, mitogens or alloantigens in mixed leukocyte cultures (MLC) rapidly gain DR antigens [1] of responder type which are synthesized by the activated cells [2, 3] and retained for at least 10 days [4]. Lymphocyte proliferation (LP) in primary or secondary MLC can be specifically blocked by antibodies directed against HLA-DR antigens of the stimulating cells [5, 6], giving rise to the contention that these serologically detected antigens are intimately related to the lymphocyte stimulating structures.

It has been reported that DR^+ activated cells from MLC of 6- or 10-day duration specifically stimulate primary and secondary HLA-D-directed LP [7–11] which can be blocked by the relevant anti-DR antisera. This is a property not shared by resting DR^- T cells. Continuously cultured interleukin 2 (IL 2)-dependent T cells express high levels of DR antigens [12] and might thus also be expected to stimulate LP. In this report, it is shown that cloned or uncloned cultured T cells (CTC), which express both SB and DR antigens, nonetheless fail to stimulate SB- or D-specific secondary proliferative responses of cloned or uncloned primed lymphocytes.

[I 3735]

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Abbreviations: **CTC:** Cultured T cells (propagated in IL 2) **CML:** Cell-mediated lympholysis **EBV:** Epstein-Barr virus **B-LCL:** B lymphoid cell line **LP:** Lymphocyte proliferation **lp:** Lymphoproliferative (response) **[^{3}H]dThd:** Tritiated thymidine **IIF:** Indirect immunofluorescence **IL 2:** Interleukin 2 **MLC:** Mixed leukocyte culture **PBMC:** Peripheral blood mononuclear cells **PLT:** Primed lymphocyte typing (line)

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2 Materials and methods

2.1 MLC and primed lymphocyte typing reagents

Peripheral blood mononuclear cells (PBMC) were isolated from normal heparinized blood by centrifugation over Lymphoprep (Nyegaard, Oslo, Norway). HLA-DR antigens were typed on nylon wool-adherent cells. HLA-Dw typing was performed with a panel of local and exchanged homozygous typing cells. Phenotyping for products of the SB gene system was undertaken using the same set of reagents initially used to establish this new HLA-linked locus [13, 14]. Bulk MLC were established between 20 Gy X-irradiated stimulators and HLA-A, B, C, SB-matched, D/DR-mismatched responders in tissue culture flasks with medium (RPMI 1640, 25 mM HEPES, 100 µg/ml gentamycin and 15% heat-inactivated pooled human serum). Activated cells were removed from culture after 6 days (MLC cells) or 10 days [primed lymphocyte typing (PLT) cells] for use as responders or stimulators.

2.2 Cloning and IL 2-dependent expansion of activated lymphocytes

Cloning was performed by limiting dilution in the presence of filler cells and conditioned medium of mitogen-stimulated human lymphocytes as a source of IL 2 [15], obtained from the Biotest Co., Frankfurt, FRG (Lymphocult T). Cloning and culture procedures for D-specific and SB-specific reagents have been fully described [16–18].

2.3 Cell-mediated lympholysis (CML) assay

Standard procedures were employed to measure CML on ^{51}Cr-labeled targets. Briefly, target cells were labeled with $Na_2{}^{51}CrO_4$ [Amersham-Buchler, Frankfurt, FRG, spec. act. 600 mCi/mg ^{51}Cr (= 22.2 GBq/mg)] washed, and distributed into U-form microtiter plates (2×10^3/well) and effector cells added in tripling dilutions to give 4 effector : target ratios. After 4 h at 37 °C an aliquot of supernatant from each well was

removed to measure specific isotope release, calculated from median values of triplicate cultures as follows:

$$\text{Release} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100.$$

Results were reduced to an expression of cytotoxicity in lytic units/10^7 effector cells, calculated by linear regression analysis of percent specific ^{51}Cr release plotted against the log of effector : target ratios. One lytic unit was defined as the number of effector cells required to achieve 25 percent specific ^{51}Cr release.

2.4 Surface marker phenotyping and detection of HLA-DR

Selected monoclonal antibodies against cell surface antigens were employed in indirect immunofluorescence (IIF) as follows: YD1/63.HLK, TÜ35 [19], and Ia NE1011 (New England Nuclear Corp., Boston, MA) were used to detect monomorphic determinants of human Ia-like antigens; NA 1/34.HLK, detecting the thymus antigen HTA1, a marker for immature T cells [20], was a kind gift of Dr. C. Milstein, Cambridge, GB; T28, detecting the mature T cell marker UCHT1 [21], was a kind gift of Dr. P. Beverley, London, GB; Lyt-3, detecting a polypeptide associated with the receptor for sheep erythrocytes (New England Nuclear); OKT3 (specific for 90–95% of mature T cells), OKT4 (detecting putative "helper/inducer" T cell markers) and OKT8 (detecting putative "suppressor/cytotoxic" T cell markers) were purchased from Ortho Pharmaceutical Corp., Raritan, NJ.

3 Results

3.1 Surface markers of alloactivated cells

3.1.1 Activated T cells express HLA-DR antigens

Surface marker phenotyping by IIF was undertaken on cryopreserved cell preparations which were to be used as stimulating cells in the following experiments. Thus T cells of different ages could be tested comparatively within a given experiment. Included as controls were nylon wool-separated T cells and B cells from the same donor blood. Cytocentrifuge preparations indicated that recovered cells were over 90% lymphocytes, and IIF confirmed that under 2% of the nylon-nonadherent cells expressed surface immunoglobulin. The results shown in Table 1 confirm that freshly prepared T cells express small amounts of HLA-DR antigens, [whereas the B cells had over 86% YD1/63.HLK-reactive cells (data not shown)], lacked the thymus antigen HTA1, and expressed high levels of the T cell antigens detected by Lyt-3, T28 and OKT3 antibodies. After 6 days in MLC, expression of DR antigens detected by all three monoclonal antibodies rose to 35–40%; after 10 days this had increased to 60–70%. The remaining populations tested derived from 6-day-old MLC-activated cells cultured for 15, 33, 45 and 81 days in IL 2 before cryopreservation. Virtually 100% of these cells expressed HLA-DR antigens, the older cells being exclusively DR^+. Mature T cell markers and the ratio of $OKT4^+ : OKT8^+$ cells remained constant during this culture period. In addition to these CTC, cloned lines were examined after isolation from MLC by limiting dilution followed by IL 2-dependent expansion of clonal progeny. Clone 29-4 (with PLT function) and clone 38-15 [with lymphoproliferative (lp) suppressor function] both expressed high levels of DR antigens and mature T cell markers. Clone 29-4 had the expected $OKT4^+/OKT8^-$ phenotype, but this was shared also by clone 38-15. Neither clone expressed HTA1 antigen.

3.1.2 Activated T cells express SB antigens

Gene products of the HLA-linked SB system are preferentially expressed on B cells rather than resting T cells. The SB antigens resemble HLA-D-region antigens in their stimulation of secondary lymphoproliferative and cytotoxic responses *in vitro*. It was thus investigated whether SB antigens, like DR antigens, appeared on activated T cells. Since SB antigens currently can be detected only by using cellular reagents, CML assays were employed to answer this question. A set of SB-

Table 1. Cell surface phenotypes of CTC

	Fresh T cells	MLC 6	PLT 10	CTC 15	CTC 33 days of culture	CTC 45	CTC 81	Clone 29-4 35	Clone 38-15 39
Antibodies detecting DR-antigens									
YD1/63.HLK	6[a]	35	65	87	87	88	100	100	100
Ia NE1011	6	35	71	84	95	100	100	100	100
TÜ35	5	39	62	63	89	92	95	95	100
Antibodies detecting mature T cells									
T28	84	86	100	100	97	100	100	100	94
Lyt-3	92	96	100	100	100	100	100	100	99
OKT3	95	97	96	100	NT[b]	100	100	96	100
OKT4	77	69	77	82	NT	80	50	100	100
OKT8	19	25	19	32	NT	20	33	0	0
Antibody detecting HTA 1									
NA1/34.HLK	0	0	0	0	NT	0	0	0	0

a) Percent cells positive in IIF.
b) NT = not tested.

specific IL 2-propagated reagents was used as effector cells in CML where target cells were CTC-derived from donors of known SB type. The level of lysis on the EBV-LCL KRO, included as a positive control [13], was higher than on the T cell targets. Nonetheless, results shown in Table 2 indicate an SB-specific lysis of CTC by these reagents, consistent with the expression of SB antigens on activated T cells. In contrast, normal T cells which had not been activated and cultured in IL 2 were very weakly and unspecifically lysed.

3.2 Lymphocyte stimulation by alloactivated 6-day MLC and 10-day PLT

3.2.1 Alloactivated cells from 6-day or 10-day cultures stimulate primary lp responses in an HLA-D-restricted fashion

Lymphocytes from 6-day-old MLC or 10-day-old PLT cultures, possessing the surface phenotype depicted in Table 1, were X-irradiated (30 GY) and titrated against a fixed number of normal PBMC to test their stimulating ability in primary MLC. The results confirmed that both 6-day-old and 10-day-old alloactivated populations were capable of stimulating HLA-D-different but not HLA-D-compatible lymphocytes (data not shown).

3.2.2 Activated cells from 10-day-old PLT stimulate specific secondary lp responses directed against either HLA-D or SB antigens

The ability of 10-day-old PLT cells specifically to restimulate HLA-D- or SB-sensitive reagents was tested in titration and kinetic experiments, in which X-irradiated stimulating cells were titrated against a fixed number of primed responding cells, and proliferative responses of replicate experiments measured after 42, 66 and 90 h coculture. Fig. 1 shows results obtained when using 10-day-old PLT cells to restimulate the SB 2-specific PLT reagent designated 193-2A [14]. Responses caused by either specific PLT cells or PBMC stimulators were equally high after 90 h coculture, although earlier responses to PBMC than to PLT cells were observed. Specificity of restimulation at autologous, third-party and specific levels was retained by PLT cell stimulation with discrimination comparable to that of PBMC restimulation. Very similar results were obtained using responders primed against other SB- or HLA-D-associated specificities (data not shown). Thus it may be concluded that DR⁺ responder cell populations obtained after 10 days PLT present not only HLA-D, but also SB antigens in a lymphostimulatory manner.

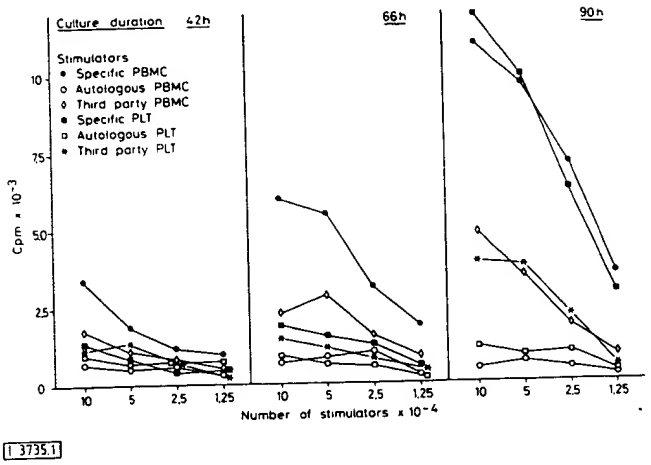

Figure 1. Kinetic study of restimulation of SB2-specific PLTL 193-2A with 10-day-old PLT cells or with PBMC. Cells from replicate experiments were harvested at different times to follow the time course of the response of the SB2-specific reagent 193-2A after restimulation with different numbers of specific ($SB2^+$), autologous ($SB1,4^+$) or third-party ($SB4,5^+$) PBMC or 10-day-old PLT cells. The number of responders was constant at 2×10^4/well. For clarity of presentation error bars have been omitted.

3.3 IL 2-propagated CTC have lost stimulatory capacity

3.3.1 IL 2-propagated CTC fail to stimulate primary lp responses

Data displayed in Table 3 show that IL 2-propagated CTC were able to respond to alloantigens but failed to stimulate strong primary lp of normal or cultured T cells. In addition to uncloned CTC, which can have a strong suppressive effect when added after irradiation to MLC [15], the ability of cloned CTC to stimulate was tested. Clone 29-4, with the surface marker phenotype shown in Table 1 and known not to suppress primary MLC, nonetheless failed to stimulate LP. Similar lack of stimulation was observed using the clone 38-15, which does suppress MLC.

3.3.2 IL 2-propagated CTC fail to stimulate secondary lp responses

The ability of IL 2-propagated uncloned as well as cloned CTC to stimulate secondary responses of HLA-D- or SB-specific reagents was next tested. Fig. 2a shows that neither uncloned CTC or PLT-functional clone 29-4 cells nor suppressor clone 38-15 cells, all of which expressed DR2, were able to restimulate the HLA-D2-specific reagent 49-3; specific PBMC as control, on the other hand, gave the expected strong restimulation responses. Stimulation by CTC was not observed at any time

Table 2. SB is expressed on activated T cells as detected in CML

Target	LCL[a]	CTC[a]	CTC	CTC	T cells[a]
SB type[b]	2,4	2,4	4,5	2,–	2,4
Effectors					
1B[c]	7.0[d]	<0.1	<0.1	<0.1[e]	<0.1[e]
2A	705	113.7	<0.1[e]	110.2	3.2[e]
2B	1786	286.1	<0.1[e]	1349	9.5
3A	9.4	14.5[e]	<0.1[e]	<0.1[e]	19.8[e]
4B	737	207.6	672	<0.1[e]	19.9[e]
5A	67.9	<0.1[e]	756	<0.1[e]	<0.1[e]

a) Target cells from the same donor KRO (7 W HTC No. 569 and 8 W HTC No. 141).
b) SB type as determined by PLT typing of donor PBMC.
c) IL 2-propagated SB-specific reagents detecting SB1 through 5.
d) Cytotoxicity given in lytic units (25%) per 10^7 effectors.
e) Regression coefficient < 0.9.

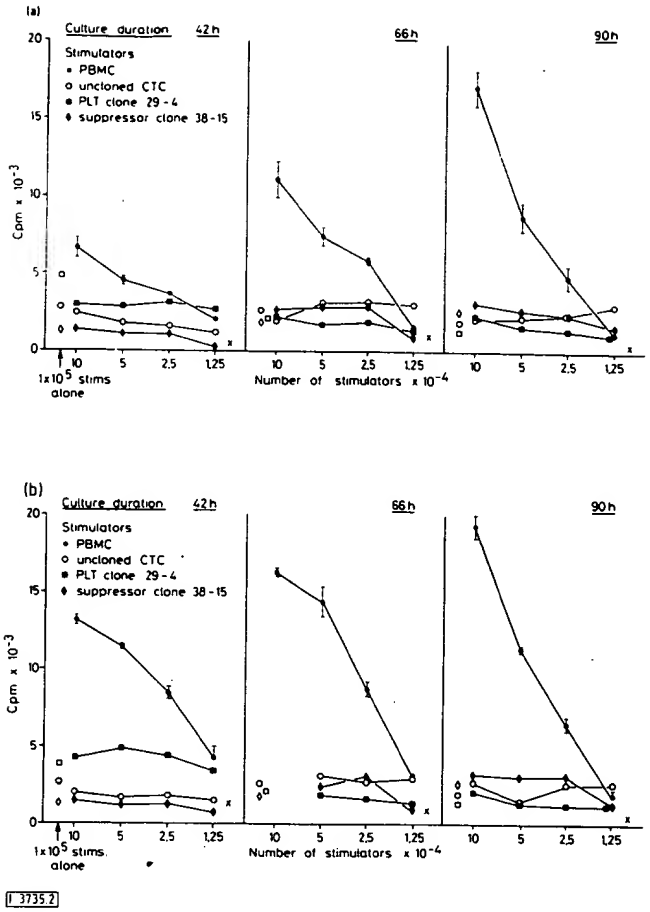

Figure 2. (a) Kinetic study of restimulation of HLA-Dw2-specific PLTL 49-3 with cloned and uncloned cultured T cells. Stimulating cells (stims.) in this kinetic study were HLA-Dw2$^+$ PBMC, or alloactivated CTC derived from this donor. [^{3}H]dThd incorporation by 1×10^5 stimulating cells in the absence of responding cells is indicated on the figure (1×10^5 stims. alone). [^{3}H]dThd incorporation by the responding cells in the absence of stimulating cells is depicted by ×. Error bars show standard error of mean for triplicate cultures. The number of responders was constant at 2×10^4/well. (b) Kinetic study of restimulation of SB2-specific PLTL 193-2A with cloned and uncloned cultured T cells. The stimulating cells in this kinetic study were derived from the same SB2$^+$ donor. [^{3}H]dThd incorporation by responding cells alone is shown as ×. The number of responders was constant at 2×10^4/well.

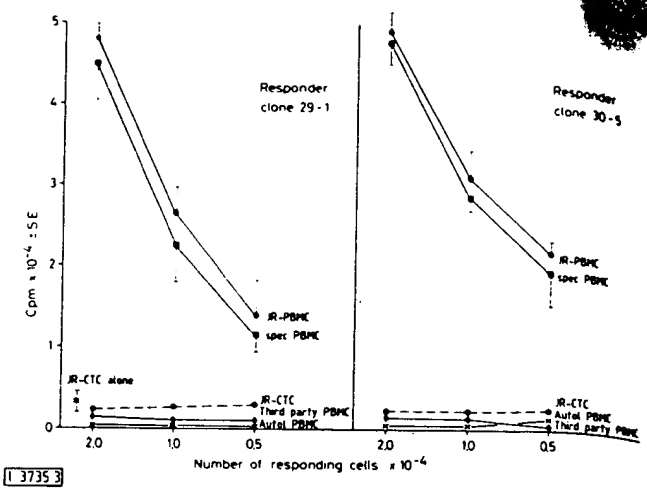

Figure 3. Cultured T cells expressing HLA-DR antigens do not stimulate cloned PLT reagents. Clones 29-1 and 30-5 are finely specific for restimulation by HLA-D5-associated determinants. The graph shows their restimulation by the original priming cell (spec. PBMC), by a donor expressing HLA-Dw5 (JR-PBMC) and by CTC derived from this donor (JR-CTC). In addition, autologous and third-party PBMC are included. All stimulating cells were used at 5×10^4/well in triplicate cultures.

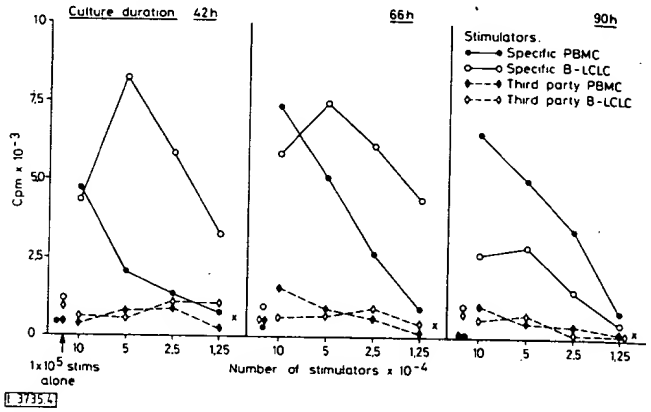

Figure 4. Kinetic study of restimulation of SB2-specific clone 62-7 with normal PBMC or B-LCL. Stimulating cells in this kinetic study were SB2$^+$ PBMC or EBV-LCL (specific) and SB4$^+$ or EBV-LCL (third-party), tested on the SB2-specific clone 62-7. The number of responders was constant at 2×10^4/well.

Table 3. Failure of IL 2-propagated CTC to stimulate primary lp responses

Responding cells[b]	Stimulating cells[a]								
	MLC 6	PLT 10	CTC 33	CTC 81	Clone 29-4	Clone 38-15	A CTC	B CTC	Dw1HTC PBMC
		days							
A PBMC	20429[c] ±1982	25637 ±2431	1421 ±265	1018 ±321	946 ±140	1316 ±162	828 ±212	1074 ±163	29527 ±2014
A CTC	18632 ±1621	24289 ±1947	1002 ± 83	1126 ±112	428 ± 58	1119 ±247	1004 ±260	2428 ±821	32728 ±8421
B PBMC	27375 ±3207	30119 ±1841	2322 ±241	1746 ±621	1016 ± 98	2019 ±120	1564 ±318	1554 ±110	46321 ±2128
B CTC	28562 ±2000	31447 ±3852	1848 ± 92	1999 ±321	821 ±192	1416 ±16	1464 ±481	2385 ±422	50454 ±6289

a) Stimulating cells at 5×10^4/well were derived from the same donor as shown in Table 1, with the exception of the Dw1 HTC cells and the A and B CTC. All were irradiated with 30 Gy prior to culture for 144 h with the responding cells.
b) Responding cells at 5×10^4/well were from donors known to be HLA-D-incompatible with the donors of the stimulating cells. Donor A CTC were 15 days old, donor B CTC were 39 days old. These cells were also irradiated and used as stimulators, for additional controls.
c) Data are presented as mean cpm of triplicate cultures ± standard deviation of the mean.

of assay, nor at any stimulating cell dose tested. Not only were these DR+ and SB+ uncloned and cloned CTC unable to restimulate HLA-D-specific PLT reagents, but as shown in Fig. 2b they also failed to stimulate the SB2-specific reagent 193-2A. Similar results have been obtained using PLT responders specific for other HLA-D and SB types (data not shown). Moreover, cloned PLT responders were also refractory to restimulation by CTC, an example of which for two different HLA-D5-specific clones is shown in Fig. 3. This was also the case with SB-specific cloned responders (data not shown).

In contrast to the failure to stimulate of strongly DR+ IL 2-propagated T cells, strongly DR+ and SB+ Epstein-Barr virus-(EBV)-transformed LCL restimulated HLA-D- or SB-specific cultured PLT reagents very well. This is illustrated in Fig. 4 using a cloned responder specific for SB2. Similar results were obtained with uncloned reagents specific for HLA-D as well as HLA-SB antigens (data not shown).

Fewer B lymphoid cell line (B-LCL) cells than PBMC were required to give maximum responses, both for HLA-D- and SB-specific responders. Larger numbers of B-LCL were inhibitory. In addition, the responses to B-LCL cells seemed to peak earlier and terminate earlier. If these considerations are taken into account, very good restimulation of both D- and SB-specific reagents by B-LCL cells can be obtained. This suggests that culture procedures themselves, which would be expected to select against nonlymphoid populations having accessory cell function, are not responsible for the failure of T lymphocytes to stimulate.

4 Discussion

It is clear from a number of reports that HLA-DR+ cell populations from 6-day or 10-day MLC, consisting mostly of alloactivated T cells, stimulate LP, and may even present antigen to helper cells [7–11]. In contrast, it has been noted that IL 2-propagated T cells, though strongly positive for DR antigens [12], seem to be unable to activate allogeneic lymphocytes [15, 22]. This apparent discrepancy has been investigated herein, and the results were as follows: 6-day-old MLC and 10-day-old PLT cells consisting predominantly of T lymphocytes with about 35% and 65%, respectively, of cells staining strongly in IIF with monoclonal anti-DR antibodies (Table 1) were clearly capable of stimulating proliferation of allogeneic but not autologous lymphocytes in primary MLC, and of specifically restimulating both HLA-D- and SB-specific secondary lp responses. In contrast however, 15- to 81-day-old IL 2-propagated CTC, containing in most cases nearly 100% DR+ T cells and expressing SB antigens, shown by their susceptibility to SB-specific CML, were unable to stimulate primary lp responses, nor secondary responses of HLA-D- or SB-specific cloned or uncloned responders. Kinetics experiments and stimulating cell titrations excluded the possibility that different culture durations or numbers of cells were required to visualize a response. Lack of essential accessory cell population for secondary responses as an explanation for this failure was made unlikely by the finding that EBV-transformed B-LCL cells were excellent stimulators of anti-D and anti-SB reagents. Moreover, the addition to the restimulation cultures of surface-adherent cells derived from the responder has not been observed to reconstitute a response (Pawelec, unpublished results).

It was necessary to be aware of the remarkable suppressive capacity, at least in primary MLC, of MLC-derived uncloned IL 2-propagated CTC [15], which, under certain circumstances, might be stimulatory but at the same time more strongly suppressive, thus obscuring responses. However, even using cloned PLT-reactive cells as stimulators, which were known not to suppress MLC [23], no positive responses were elicited in primary or secondary MLC. Therefore it seems clear that suppressor mechanisms were not masking stimulatory activity. These data indicate that strongly DR+ T cells are indeed intrinsically incapable of stimulating LP. Results of current experiments aimed at attempting to reconstitute the ability of such CTC to stimulate by adding purified exogenous IL 1 and/or IL 2 may be important in answering the question of why these DR+ and SB+ cells fail to deliver a complete proliferative signal. Alternatively it may be that cells initially responsive to IL 2 belong to a subset of T lymphocytes intrinsically incapable of triggering lp responses despite their expression of Ia-like antigens.

Based on these results one must consider the question of why 6-day-old MLC or 10-day-old PLT (non-IL 2-cultured) cell populations do stimulate. Of particular interest in this regard is a recent report [24] that the lymphocyte stimulatory population contained in 6-day-old MLC cells is in fact a DR+ "dendritic-like" non-T cell and that the T cells, even in 6-day MLC preparations, themselves do not stimulate. Such dendritic cells in mice and man are known to express large amounts of Ia antigens and to be extremely potent stimulators in MLC [25, 26]. The data reported here may indicate that, in addition, they express SB antigens. The reason that populations of CTC fail to stimulate would then be that they simply lack this subpopulation of dendritic-type cells, which do not respond to growth factors, and die out in prolonged culture.

Since Ia-like antigens on activated T cells seem to be incapable of stimulating typical LP, the question of their distinct functional properties remains to be answered. It might be that such T cell DR and SB antigens could still possess a special lymphocyte stimulatory capacity, for example highly restricted in the nature of the responders that could be activated in autologous regulatory lymphocyte circuits. This hypothesis is amenable to ongoing investigations using cloned T cells of different defined functions as responders and stimulators. Certainly, little evidence exists to date to suggest that DR+ CTC can stimulate proliferation of autologous PBMC or autologous uncloned CTC (Table 3, [15]). Even occasional low-level stimulation apparently caused by CTC may be attributable to "carryover" of residual mitogen from the conditioned medium by the cultured stimulating cells [15]. However, the possibility also remains that certain responding clones could be affected by DR or SB antigens in a way not detectable in the [^{3}H]thymidine ([^{3}H]dThd) incorporation assay, because they are too rare in heterogeneous populations, or because they do not proliferate. Pure populations of DR+ and SB+ human T cells should thus be valuable in further dissection of the mechanism of lymphocyte stimulation and the regulation of proliferation.

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Antibody-specific immunoregulation is restricted by the major histocompatibility gene complex*

Experiments were carried out to examine the major histocompatibility complex (MHC) restriction of an antibody-specific immunoregulatory function that develops following conventional immunization with hapten-carrier conjugates. It was found that carrier-primed hosts reconstituted with Ly-2.2$^+$ T cells from hapten-carrier-immunized donors were able to suppress adoptively transferred primary B cells. The effective suppression of primary B cell responses was dependent on syngeny between the suppressing T cell population and the target B cells at both MHC and Igh. These findings indicate that T cells that function through the recognition of B cell antibody are restricted in their function by gene products of the MHC, similar to the restrictions observed for the recognition of conventional antigens.

1 Introduction

In recent years, evidence has accumulated indicating that T cells recognize antigen in the context of molecules encoded in the major histocompatibility complex (MHC). Helper T cells require the recognition of I region-encoded molecules [1, 2], whereas cytotoxic T cells are restricted to the recognition of molecules encoded in the K, L, or D loci [3]. This dependency on the recognition of MHC-encoded gene products suggests that these molecules play a pivotal role in selecting T cell specificities during the generation of an individual's T cell repertoire. However, several recent reports suggest that not all regulatory T cell functions are restricted by the MHC and that other molecules, in particular immunoglobulin (Ig), may serve as restricting elements in immune responses. Bottomly and coworkers found that antigen-specific helper T cells, essential in the triggering of TEPC 15$^+$ B cells in response to phosphorylcholine, do not require the recognition of MHC-encoded molecules [4, 5]. Yamachi et al. have presented evidence that certain antigen-specific T cells in suppressor pathways require identity with target cells at the Ig heavy chain (Igh) locus but not at MHC [6]. Similarly, Juy et al. have shown that suppressor T cells, specific for the MOPC 460

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Abbreviations: **BSA:** Bovine serum albumin **DNP:** 2,4-Dinitrophenyl **Hy:** Hemocyanin **MHC:** Major histocompatibility complex **NP:** (4-Hydroxy-3-nitrophenyl)acetyl **C:** Complement

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